1991

Biochemical And Morphological Characterization Of Cytoplasmic Particles From Avian Reticulocytes

Timothy W. Blaker

BIOCHEMICAL AND MORPHOLOGICAL CHARACTERIZATION OF CYTOPLASMIC PARTICLES FROM AVIAN RETICULOCYTES

by

Timothy W. Blaker

Department of Zoology

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario
July 1991

ISBN 0-315-66331-6

ABSTRACT

Ferritin can be isolated from quail reticulocyte lysates using a rapid two-step purification method that combines heat denaturation and proteinase-K treatments to produce high yields of pure, undegraded ferritin. Ferritin remains in the supernatant of heat-denatured samples, and subsequent treatment with proteinase-K eliminates non-ferritin proteins that remain in the supernatant as well as non-ferritin proteins that are found in association with ferritin.

Ferritin is a heat shock protein of quail reticulocytes. Its synthesis in heat-shocked cells is 3-5 times greater than in control cells. Ferritin subunits synthesized in response to heat shock consist of isoforms that are identical in size and pI to those present in ferritin synthesized in control cells. The mechanism of this increased synthesis involves translational control.

The "prosome" fraction isolated from quail reticulocyte lysates by sucrose density gradient ultracentrifugation contains two biochemically and morphologically distinct particles. The major constituent (~95%) of this fraction is the proteasome, an M_r ~562,000 particle that possesses proteolytic activity. The proteasome is a cylinder ~15 x 10 x 10 nm, and is composed of a set of protein subunits ranging in size from ~22-~32 kDa. One of the subunits of the proteas-

ome cross-reacts with a monoclonal antibody to prosome protein P27. The proteasome is composed of a spherical central component (~10 nm diameter) enclosed by two, 2.5 nm thick hexagonal end caps. The prosome particle is formed by degradation of the proteasome particle during sample preparation for electron microscopy. The minor constituent (<5%) of the prosome fraction is ferritin, a 12-13 nm diameter particle composed of 21 kDa subunits, which is found as the protein component of an M_r ~877,000 particle.

Ferritin is also the protein component of the ~35S "prosome-like-particle", where it is found in association with small RNAs (~80 nucleotides in length) that hybridize to a globin cDNA.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank the large number of people who have helped and guided me over the course of this study; I couldn't have done it without them.

Thank you to my Supervisor, Dr. Burr Atkinson, for giving me the opportunity to do this work; and to my Advisory Committee: Dr. Robert Cook, Dr. Rob Dean, and Dr. Michael Locke, for all their interest, suggestions, and support.

I'd like to especially thank Dr. Locke for the use of his electron microscope; and to thank Jackie Sparks and Harry Leung for their technical assistance.

Thanks to Dr. Ron Podesta for the use of his laser printer; and to Dr. Barbara Bain for getting me started on the department EM.

Thanks to Reg Johnston of the Department of Plant Sciences for letting me use the gradient fractionator in his lab on those occasions when I was unable to get ours working.

Thanks to Alex Sangster and Dan McGregor for maintaining the quail colony.

I'd like to say thanks to Roger Frappier for all the little things he did for me; and to Dr. Cheryl Ketola for her interest and amusement.

I must say thank you to Dr. Dick Shivers for all the times he lent me his sets of Allen keys and his circuit meter (even though I usually hung on to them for too long).

Thank you to my parents, Pat and Betty Blaker, for all their support and encouragement.

And the most special thank you's of all ... to my wife Sandy, who has always supported me, and to Dana, Brian, and Sara: Daddy hasn't been much fun these last several months, but I promise to make it up to you!

Technical acknowledgements

The electron micrographs presented in Chapters 1 and 2, and some of the electron micrographs presented in Chapter 3 were taken by Dr. Michael Locke, Department of Zoology, University of Western Ontario. The protease assay referred to in Chapter 3, Table 3.1 was done by Dr. R.A. Cook, Department of Biochemistry, University of Western Ontario. The image enhancement presented in Chapter 3 was done by Harry Leung, Department of Zoology, University of Western Ontario. The RNA hybridization presented in Chapter 3 was done by Caren Helbing and Manish Raizada. I am very grateful to these people for their excellent technical assistance.

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CHAPTER 1 - The rapid purification of ferritin from lysates of red blood cells using proteinase-K

1.1 INTRODUCTION

Ferritin, or holoferritin, is the major iron-storage protein in animal cells (Theil, 1983). The iron-free protein moiety of ferritin, apoferritin, has a relative molecular mass of approximately 450,000 and is composed of 24 subunits (Munro and Linder, 1978). These subunits are arranged in 12 dimers, and each dimer is held together by hydrophobic interactions between the regions from amino acid residues 73 to 91 of each subunit; interactions between the hydrophobic region from residues 160 to 169 with the same region of other dimers permits the assembly of the apoferritin shell (Ford et al., 1984). The end result is a very stable protein shell that can withstand a variety of harsh treatments including: heating at 80°C., incubation in 10M urea or 1% SDS, as well as extremes of pH (Ford et al., 1984).

The exceptional stability of ferritin at elevated temperatures (Hofmann and Harrison, 1963; Listowsky et al., 1972) is often exploited as a first step in purification. Heating of cell lysates or tissue homogenates, prepared in water or in a low salt buffer, for 10 minutes at 70-80°C followed by cooling, results in the coagulation of most proteins and the retention of ferritin (both holo- and

apoferritin) in solution. Ammonium sulfate precipitation of the heat-resistant ferritins followed by gel permeation chromatography usually gives a more purified, but still contaminated, preparation of the ferritins (Linder and Munro, 1973; Munro and Linder, 1978). Other purification steps, such as $CdSO_4$ precipitation (Granick, 1951), ion-exchange chromatography (Drysdale and Ramsey, 1965; Pagé et al., 1980), immunoprecipitation (Linder-Horowitz et al., 1969; Munro and Linder, 1978), acetone precipitation (Kato and Shimada, 1970), high-speed centrifugation (Crichton et al., 1973), immunoabsorption (Worwood et al., 1976), or heating of a methanol-treated lysate (Cham et al., 1985), have been used by various workers in the final stages of ferritin isolation. These methods are tedious, result in considerable loss of the protein, and, in some cases, can discriminate by selecting the iron-containing (holo) or the iron-free (apo) ferritins and/or favour the isolation of selective subpopulations of the ferritins (Linder-Horowitz et al., 1969; Gonyea et al., 1976, Munro and Linder, 1978; Aisen and Listowsky, 1980; Pagé et al., 1980).

This study was undertaken after the resistance of commercially available holo- and apo-ferritins to brief digestion with trypsin, papain, or pepsin (Granick, 1946; Tria and Torboli, 1948; Mazur et al., 1950; Harrison and Hofmann, 1962) was confirmed, and it documents the resistance of quail reticulocyte ferritin to the proteolytic activity of proteinase-K.

Proteinase-K is a serine protease that randomly cleaves polypeptides by hydrolyzing peptide or ester bonds adjacent to the carboxyl group of aromatic or hydrophobic aliphatic amino acids. It is stable over a wide pH range (4-12.5), and is more active at elevated temperatures and on denatured protein (12X more active at 65°C; 7X more active in the presence of SDS). Its most common use is to rapidly inactivate endogenous nucleases (RNAses and DNAses) prior to the isolation of native RNA and DNA by phenol extraction (Sambrook et al., 1989).

The resistance of ferritin to proteinase-K treatment, resulting from the inaccessibility of suitable sites on the native protein, in conjunction with its resistance to heat denaturation, makes it possible to rapidly isolate high yields (>90%) of pure, undegraded ferritin from the postmitochondrial supernatant of quail reticulocyte lysates.

1.2 MATERIALS AND METHODS

1.2.1 Blood collection and radio-labelling of protein

Adult (~3 month-old) Japanese quail (Coturnix coturnix japonica) were made anemic by three successive daily subcutaneous injections of neutralized phenylhydrazine hydrochloride (15 mg/kg). Two days after the third injection, birds were killed by decapitation and blood was collected into 15 ml conical bottom tubes (Falcon 2095; Becton Dickinson, Lincoln Park, NJ) containing 0.1 ml Hepalean (Organon Canada, Toronto, ONT). The blood cells were enriched for reticulocytes by pelleting through Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) by the method of Dean and Atkinson (1985). The reticulocytes were resuspended in 75 cm^2 Tissue Culture Flasks (Corning Glass Works, Corning, NY) containing methionine-free minimum essential medium (1X MEM, with Earle's Salts and sodium bicarbonate, without methionine, without glutamine; GIBCO, Grand Island, NY), at a concentration of ~0.3-1.2 x 10^6 cells/ml, and incubated at 37° for 30 minutes. The cells were radioactively labelled by the addition of 60 μCi/ml of L-^{35}S-methionine (Dupont-New England Nuclear, Boston, MA; specific activity >1000 Ci/mmole). Cells were labelled for 2 hours at 37°, with agitation of the flasks at 30 minute intervals.

1.2.2 Production of a postmitochondrial supernatant

Labelled cells were collected in 50 ml conical bottom tubes (Falcon 2098; Becton Dickinson) and pelleted at 100 x g for 10 minutes. The cells were washed in 5 volumes of methionine-free medium and pelleted at 100 x g for 10 minutes. The labelled cells were lysed in 5 volumes cold (4°C) lysing buffer (LB: 10 mM Triethanolamine-HCl (TEA-HCl, pH 7.4), 10 mM NaCl, 1 mM $MgCl_2$) and left on ice for 30 minutes. The lysate was centrifuged at 20,000 x g for 20 minutes, and the postmitochondrial supernatant (PMS) was stored at 4°C.

1.2.3 Heat denaturation treatment

Aliquots of the PMS were placed in 15 or 30 ml Corex tubes (Corning Glass Works, Corning, NY) and heat-denatured by placing them at 75-80°C for 10 minutes. The treated samples were placed on ice for 30 minutes, centrifuged at 5,000 x g for 30 minutes, and the supernatants (containing nondenatured protein) were concentrated in Centriflo Type CF50A membrane cones (Amicon Corp., Danvers, MA) prior to storage (at 4° or -70°C) and analysis.

1.2.4 Proteinase-K treatment

Proteinase-K (Boehringer-Mannheim, Laval, Québec), prepared in 80 mM Tris-HCl (pH 6.8), was added to the PMS so that the final concentration of enzyme was 0.2 µg/µl (i.e. 1 µg of proteinase-K/80 µg of protein). The samples were incubated in capped tubes at 37°C for 15 minutes on a shaker bath. After the 37°C incubation, the samples were heated at 75-80°C for 10 minutes to inactivate and precipitate the denatured enzyme, and subsequently cooled on ice for 30 minutes. The cooled samples were centrifuged and the resulting clear supernatants were either transferred to separate tubes for storage or concentrated in Centriflo Type CF50A membrane cones and stored at 4°C or -70°C.

1.2.5 Measurement of protein concentration

Samples were assayed for protein concentration by the method of Lowry *et al*. (1951) using bovine serum albumin, ferritin, or apoferritin as standards.

1.2.6 Measurement of radioactivity

Radioactively-labelled protein in aliquots of PMS was precipitated by the addition of 1/3 volume 50% trichloroacetic acid (w/v). Samples were mixed gently, placed on ice for 30

minutes, and centrifuged (5,000 x g, 10 minutes). The pellets were rinsed with diethylether, and recentrifuged. The ether was removed, and the pellets were dried *in vacuo* overnight. The dried pellets were resuspended in 80 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol (β-MEt), and aliquots were removed for liquid scintillation counting.

Radioactivity was measured by a Beckman LS-255 liquid scintillation counter (Beckman Instruments, Palo Alto, CA) using a Triton X-100 - toluene - Omnifluor (Dupont-New England Nuclear, Dorval, Québec) cocktail.

1.2.7 Polyacrylamide gel electrophoresis

1.2.7.1 One-dimensional separations

1.2.7.1.1 Nondenaturing separations

One-dimensional nondenaturing polyacrylamide slab gels consisted of a 3-15% (0.375M Tris-HCl (pH 8.9)) separating gel, overlaid with a 3% stacking gel (0.125M Tris-HCl (pH 6.8)). All electrophoresis chemicals were Bio-Rad Electrophoresis Grade (Bio-Rad Laboratories, Richmond, CA). Running buffer contained 25 mM Tris-HCl (pH 8.3), 192 mM glycine. Samples were made to contain 80 mM Tris-HCl (pH 6.8) by the addition of one-third volume of a 4x nondenaturing electrophoresis sample buffer (320 mM Tris-HCl (pH 6.8), 20% glycerol (w/v), 0.1% Bromphenol Blue). Samples were electrophoresed at 5 mA per gel until the dye front had moved into the separating

gel, and then at 15 mA per gel until the dye front had just run off the bottom of the separating gel. Nondenaturing electrophoresis was done in a cold-room (4°C). Gels were stained in 0.1% (w/v) Coomassie Blue G (C.I. 42655; Sigma Chemical Co., St. Louis, MO) in methanol:water:acetic acid (40:50:10), destained in methanol:water:acetic acid (10:80:10), and stored in water:acetic acid (90:10). The relative molecular mass (M_r) was estimated by comparing their distance of migration through the separating gel to a standard curve of Log (Molecular weight) (ordinate) vs Distance Migrated (abscissa) prepared using marker proteins of known molecular weight separated on the same gel (High Molecular Weight Calibration Kit: thyroglobulin - 669,000 Da; ferritin - 440,000 Da; catalase - 232,000 Da; lactate dehydrogenase - 140,000 Da; bovine serum albumin - 67,000 Da; Pharmacia/LKB, Uppsala, Sweden).

1.2.7.1.2 Denaturing separations

One-dimensional sodium dodecyl sulfate (SDS) polyacrylamide slab gels consisted of either a 3-15% or a 7.5-17.5% separating gel (0.375M Tris-HCl (pH 8.9), 0.1% (w/v) SDS) overlaid with a 3% stacking gel (0.125M Tris-HCl (pH 6.8), 0.1% (w/v) SDS). Running buffer contained 25 mM Tris-HCl (pH 8.3), 192 mM glycine, 0.1% (w/v) SDS. Samples were made to contain 80 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 5% (v/v) β-MEt,

10% (v/v) glycerol, and 0.1% Bromphenol Blue. Holo-and apoferritins were separated on linear gradient gels of 3-15% polyacrylamide, whereas ferritin subunits, generated by boiling the samples for 3 minutes in 2% SDS and 5% β-mercaptoethanol (Schaefer and Theil, 1981), were separated on linear gradient gels of 7.5-17.5% acrylamide.

Electrophoresis was done at 5 mA/gel until the dye front had moved into the separating gel, and then at 15 mA/gel until the dye front had run off. Gels were stained in 0.1% (w/v) Coomassie Blue G (C.I. 42655; Sigma Chemical Co., St. Louis, MO) in methanol:water:acetic acid (40:50:10), destained in methanol:water:acetic acid (10:80:10), and stored in water:acetic acid (90:10).

The relative molecular mass was estimated by comparing their distance of migration through the separating gel to a standard curve of Log (Molecular weight) (Ordinate) vs Distance Migrated (Abscissa) prepared using marker proteins of known molecular weight separated on the same gel (Low Molecular Weight Calibration Kit: phosphorylase b - 94,000 Da; bovine serum albumin - 67,000 Da; ovalbumin - 43,000 Da; carbonic anhydrase - 30,000 Da; soybean trypsin inhibitor - 20,000 Da; α-lactalbumin - 14,400 Da; Pharmacia/LKB, Uppsala, Sweden).

1.2.7.2 Two-dimensional separations

Two-dimensional separation of proteins was done according to the method of O'Farrell (1975) with some minor modifications (see below). Separation was achieved by isoelectric focussing in the first dimension (i.e. proteins were separated on the basis of their pI) and by SDS-PAGE in the second dimension (i.e. separated on the basis of size).

The isoelectric focussing gel was a 3% polyacrylamide tube gel containing 9M urea, 2% NP-40, and 2% ampholines (Pharmacia/LKB, Bromma, Sweden; pH 3.5 to 10). These gels were pre-run (to establish the pH gradient) at 200V for 15 minutes, 300V for 30 minutes, and 400V for 30 minutes. The upper buffer was 0.25N NaOH, and the lower buffer was 0.2N H_3PO_4. The lower buffer was cooled by a water jacket to ~10°C. After the pre-run, the upper buffer was removed from the chamber and from the tops of the tube gels, and the samples (made to contain 9M urea, 1% NP-40, 2% ampholines, and 5% β-MEt) were applied. Overlay buffer (4.5M urea, 1% ampholines) was applied, and subsequently covered with fresh upper buffer. Gels were electrophoresed at 400V for ~18 hrs, and then at 800V for 2 hrs. After electrophoresis, tube gels were extruded into a solution containing 80 mM Tris-HCl (pH 6.8), 2% SDS, and 5% β-MEt, and allowed to equilibrate for ~30 minutes (with agitation). The tube gels were adhered to the top of slab gels (either a 3-15% or a 7.5-17.5% SDS-polyacrylamide

separating gel with a 3% stacking gel) with 1% agarose (containing 80 mM Tris (pH 6.8), and 0.1% SDS) and run as previously described.

1.2.8 Staining of polyacrylamide gels for the presence of iron

Gels were stained for iron in a solution of 2% acetic acid, 0.2% mercaptoacetic acid, and 0.75 mM sodium ferene made up in glass-distilled water (Chung, 1985). Gels were photographed, stained for protein with Coomassie brilliant blue G, photographed, and prepared for fluorography (when applicable).

1.2.9 Fluorography of polyacrylamide gels

Destained gels were soaked in three changes of dimethylsulfoxide (DMSO) to remove all traces of water and acetic acid. Each gel was then impregnated with 2,5-diphenyloxazole (PPO) by soaking in a 22% (w/v) solution of PPO in DMSO. The PPO-impregnated gels were immersed in an excess of water to precipitate the PPO, and dried down on to Whatman 3MM filter paper (Bonner and Laskey, 1974) using a Bio-Rad Model 224 Gel Dryer (Bio-Rad Laboratories, Richmond CA). The dried gels were taped to stiff cardboard and apposed to Kodak X-Omat RP X-ray film (Eastman Kodak, Rochester, NY) preexposed to an absorbance (at 540 nm) of ~0.15 (Laskey and Mills, 1975). Exposure of the film was carried out at -70°C.

1.2.10 Immunoblotting analysis of quail ferritins

Electrophoretically-separated proteins were transferred (using a Bio-Rad TransBlot Cell (Bio-Rad Laboratories)) at 60V for 8 hours to nitrocellulose (DuPont-New England Nuclear, Dorval, Québec), using 25 mM Tris (pH 8.3), 192 mM glycine, 20% v/v methanol as the transfer buffer (Towbin et al., 1979). The nitrocellulose was blocked for 1 hour (at room temperature) in a solution of 3% (w/v) gelatin (Bio-Rad Laboratories) in tris-buffered saline (TBS: 20 mM Tris-HCl (pH 7.5), 500 mM NaCl). The blocked nitrocellulose was incubated in primary antibody solution containing rabbit anti-horse ferritin (shown to cross-react with avian ferritins; Theil and Brenner, 1981) (Cappel Research Products; Organon Teknika, Scarborough, ONT) diluted 1:3,000 (in TBS containing 1% (w/v) gelatin) for 8 hours at room temperature, washed twice with TBS, and incubated in secondary antibody solution containing goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad Laboratories) diluted 1:1,000 (in TBS) for 2 hours at room temperature. Following the incubation with the secondary antibody the nitrocellulose was washed twice (5 minutes each) with TBS.

Cross-reaction of primary and secondary antibodies was visualized using 4-chloro-1-naphthol (4-CN; Bio-Rad Laboratories) as a substrate for the horseradish peroxidase conjugated to the secondary antibody. A solution of 60 mg of 4-CN in 20 ml of methanol was added to a solution of 100 ml TBS

containing 60 µl 30% H_2O_2, immediately prior to use. The rinsed membranes were incubated in the resulting solution for ~30 minutes (in the dark), and colour development was stopped by rinsing in distilled water. The developed immunoblots were stored in water (in the dark) until photographed.

1.2.11 Concentration of protein by electroelution

Holo- and apo-ferritins were extracted by electroelution using an ISCO Model 1750 Sample Concentrator (Instrumentation Specialties Co., Lincoln, NE) by the method of Bhown et al., (1980) from areas in unfixed and unstained lanes of gels (corresponding to the stained areas on other lanes in the same gels). The eluants were assayed for protein and radioactivity as previously described.

1.2.12 Electron microscopy

Small drops (0.5 µl) of horse spleen ferritin (Sigma), and heat-denatured and proteinase-K-treated/heat denatured samples isolated from quail PMS were smeared across Formvar-coated, carbon-shadowed copper grids with a piece of filter paper and allowed to air dry. Preparations were negatively stained with sodium phosphotungstate (Locke and Huie, 1980) and viewed on a Philips EM 200 at 80 kV.

1.3 RESULTS

1.3.1 One-dimensional nondenaturing PAGE analysis of ferritins prepared from proteinase-K-treated PMS

Samples of the heat-denatured and the proteinase-K-treated/heat-denatured ferritins isolated from the PMS were separated by one-dimensional nondenaturing-PAGE. Samples treated with proteinase-K contain fewer extraneous proteins than samples treated by heat denaturation only (Figure 1.1, Panels A and B, lanes 1 and 2). The ferritins treated with proteinase-K also have greater mobility on native gels than ferritins prepared by heat denaturation only (Figure 1.1, Panels A, B, and C).

1.3.2 One-dimensional SDS-PAGE analysis of ferritins prepared from proteinase-K-treated PMS

Samples of the heat-denatured and the proteinase-K-treated/heat-denatured ferritins isolated from the PMS were separated by one-dimensional SDS-PAGE. The difference in the mobility of ferritin prepared by these methods (see Figure 1.1) is still evident, although less apparent (Figure 1.2, Panels A and B). Reduction of the ferritins to their subunit (monomer) components (M_r = 21,000), by boiling the prepara-

Figure 1.1. One-dimensional nondenaturing PAGE of the heat-soluble ferritins obtained from quail postmitochondrial supernatants. Lane 1, heat-denatured sample; lane 2, proteinase-K-treated, heat-denatured sample. Panel A, Coomassie-blue stain; Panel B, fluorogram of Panel A; Panel C, stained for iron. Marker proteins of relative molecular mass ($M_r \times 10^{-3}$) are indicated.

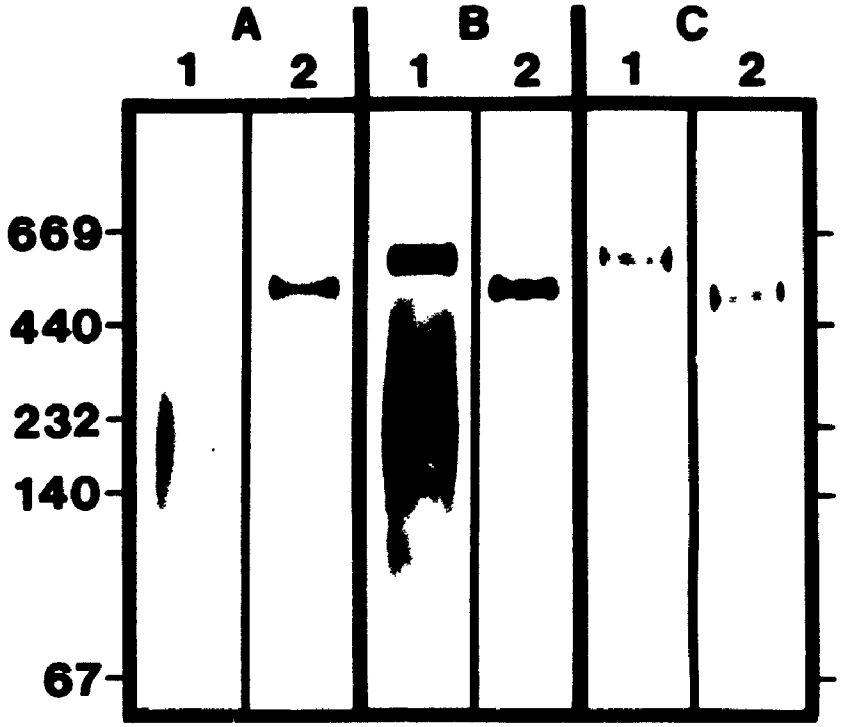
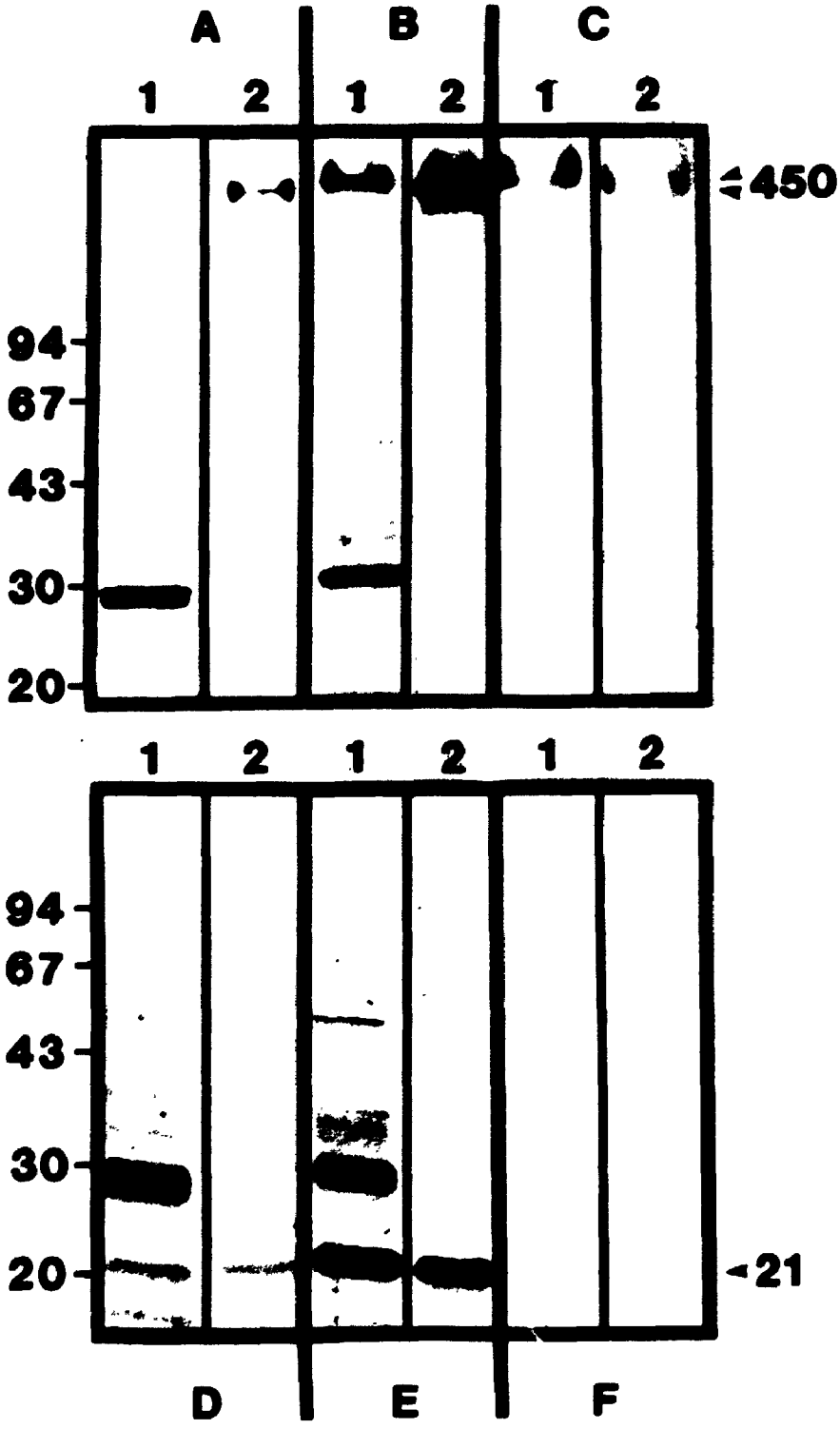

A
B
C
1
2
1
2
1
2
669
440
232
140
67

Figure 1.2. One-dimensional SDS-PAGE of heat-soluble ferritins obtained from quail postmitochondrial supernatants. Lane 1, heat-denatured sample; lane 2, proteinase-K-treated, heat-denatured sample. All samples were made to contain 2% SDS and 5% β-MEt. Panels A, B, and C, samples were not boiled prior to electrophoresis; panels D, E, and F, samples were boiled in prior to electrophoresis. Panels A and D, Coomassie-blue stain; panels B and E, fluorograms of panels A and D, respectively; panels C and F, immunoblots of gels similar to panels A and D, respectively, assayed with anti-ferritin (see Materials and Methods for details). The region from ~28 to ~50 kDa in panels A, B, D, and E, contain non-ferritin protein contaminants present in preparations not treated with proteinase-K. Arrowheads on the right-hand side denote the ferritin polymers (~450 kDa) and monomers (~21 kDa). Marker proteins of relative molecular mass ($M_r \times 10^{-3}$) are indicated.


A
B
C
1
2
1
2
1
2
450
94
67
43
30
20
1
2
1
2
1
2
94
67
43
30
20
21
D
E
F

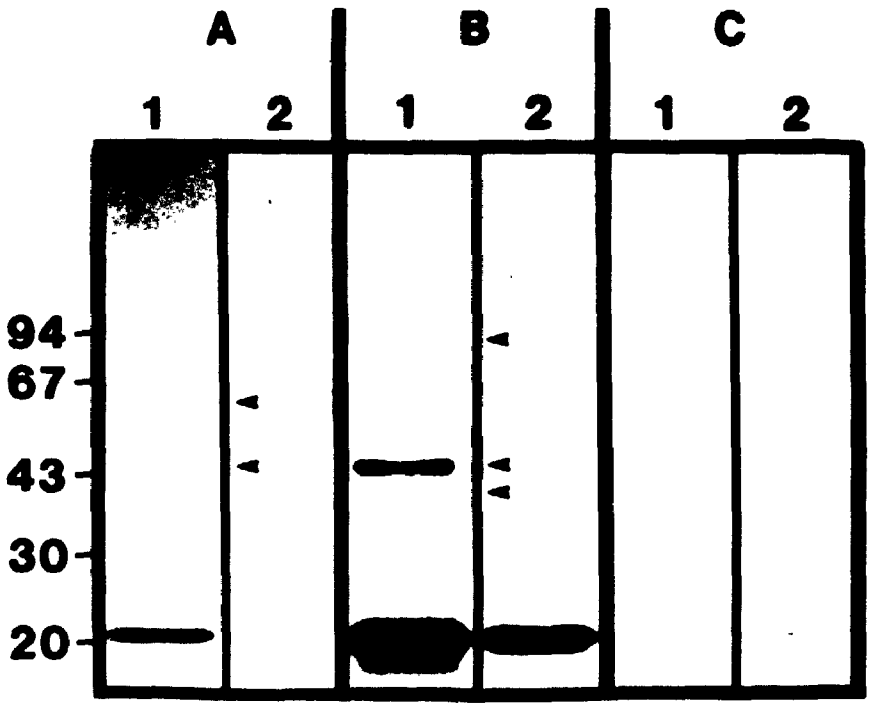
tions in the presence of 2% SDS and 5% β-mercaptoethanol prior to electrophoresis (Schaefer and Theil, 1981), indicates that the simplest components of ferritin (i.e. the 21,000 dalton polypeptides) prepared by either method have the same electrophoretic mobility on one-dimensional gels (Figure 1.2, Panels D and E).

Immunological analysis shows that the two bands identified by molecular mass as ferritin and ferritin subunits cross-react with antibodies to horse spleen ferritin (Figure 1.2, panels C and F).

1.3.3 Analysis of SDS-PAGE-separated ferritin polymers

Samples of heat-denatured and proteinase-K-treated/heat-denatured ferritins isolated from the PMS were separated by one-dimensional SDS-PAGE, and the regions containing ferritin polymers (determined by Coomassie-blue staining) were excised from unstained lanes, and the protein concentrated from them by electroelution. The ferritin polymers were converted to their monomeric forms (M_r = 21,000) by heating the samples in the presence of SDS and β-mercaptoethanol, and the samples were reanalyzed by one-dimensional SDS-PAGE. The results demonstrate that proteins that do not react with ferritin antibodies are detectable as constituents which remained associated with SDS-PAGE-separated ferritin polymers not treated with proteinase-K (Figure 1.3, panels A and B, lane 1).

Figure 1.3. One-dimensional SDS-PAGE analysis of non-ferritin proteins that remain associated with SDS-PAGE-separated ferritin polymers not treated with proteinase-K. Ferritin polymers were electroeluted from one dimensional SDS-PAGE-separated samples, converted to their monomers (M_r = 21,000) by boiling them in SDS and β-mercaptoethanol, and reapplied to one-dimensional SDS polyacrylamide gels. Lane 1, heat-denatured sample; lane 2, proteinase-K-treated, heat-denatured sample. Panel A, Coomassie-blue stain; panel B, fluorogram of panel A; and panel C, immunoblot assayed with anti-ferritin (see Materials and Methods for details). Arrowheads indicate the non-ferritin proteins that remain affiliated with SDS-PAGE-separated ferritin polymers not treated with proteinase-K. Marker proteins of relative molecular mass ($M_r \times 10^{-3}$) are indicated.


A
B
C
1
2
1
2
1
2
94
67
43
30
20

1.3.4 Two-dimensional SDS-PAGE analysis of ferritins prepared from proteinase-K-treated PMS

Samples of the heat-denatured and the proteinase-K-treated/heat-denatured ferritins isolated from the PMS were separated by two-dimensional SDS-PAGE. The ferritins isolated by either method have isoforms with the same isoelectric points (Figure 1.4).

1.3.5 Electron microscopic analysis of ferritins prepared from proteinase-K-treated PMS

The heat-denatured and the proteinase-K-treated/heat-denatured ferritins isolated from the PMS were examined by electron microscopy of negatively stained samples. The ferritins isolated from the PMS are indistinguishable from commercially available horse spleen ferritin (Figure 1.5).

Figure 1.4. Two-dimensional IEF-SDS-PAGE analysis of L-[^{35}S]-methionine-labelled ferritin subunits obtained from quail reticulocyte postmitochondrial supernatant. Panel A, heat-denatured sample; panel B, proteinase-K-treated, heat-denatured sample. Samples were boiled in the presence of SDS and β-mercaptoethanol prior to application to the first dimension IEF gel (see Materials and Methods for details). Isoelectric points were estimated from a standard curve obtained from a sample-free IEF gel that was run at the same time. Only the ~15--25 kDa region of the second dimension SDS gels are shown.

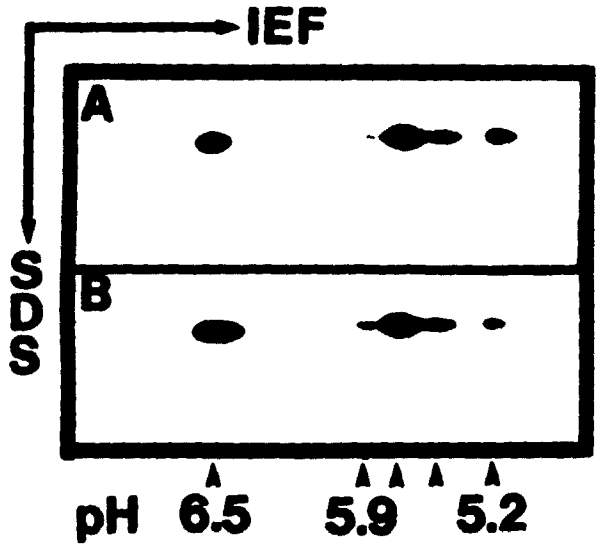
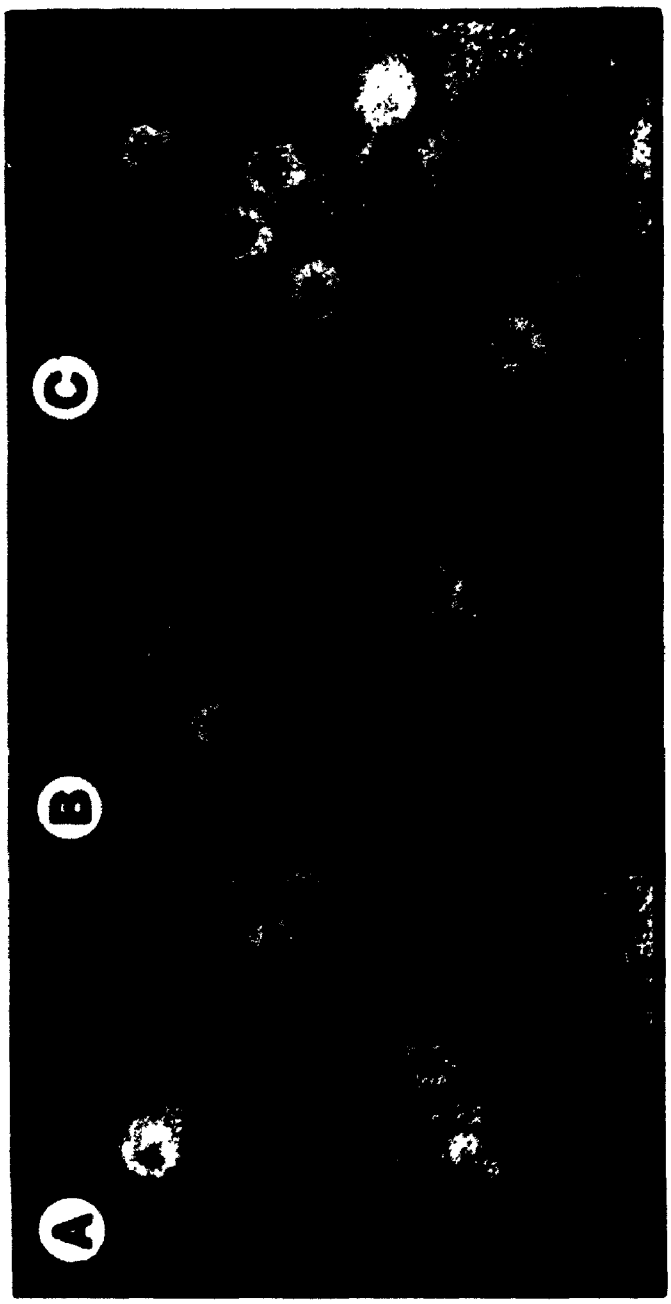

IEF
A
B
SDS
pH 6.5 5.9 5.2

Figure 1.5. Electron microscopic analysis of the ferritins obtained from quail reticulocyte PMS. Panel A, horse spleen ferritin; panel B, ferritin obtained from heat-denatured PMS; panel C, ferritin obtained from proteinase-K-treated/heat-denatured PMS. The ferritins obtained from either heat denatured or proteinase-K-treated/heat denatured quail reticulocyte PMS are indistinguishable from commercially available horse spleen ferritins. Scale bar = 20 nm.


A
B
C

1.4 DISCUSSION

In 1986, I was interested in isolating large amounts of an unidentified protein, found in quail reticulocytes, that appeared to be a previously unrecognized heat shock protein. I attempted to purify this protein by electroeluting it from excised pieces of SDS-polyacrylamide gels. This method resulted in the purification of very small amounts of protein, especially relative to the time required. At about this time I tentatively identified the protein as ferritin, and realized that it might be possible to enrich the sample by eliminating at least some of the non-ferritin components. This study documents the resistance of quail reticulocyte ferritin to the proteolytic activity of proteinase-K.

The procedure for rapidly purifying high yields of ferritin makes use of reports from the 1940s and 1950s that have demonstrated that neither holoferritin nor apoferritin are markedly digested by beef liver homogenates, trypsin (2 days at 38°C, pH 7.5), papain, pepsin (Granick, 1946; Tria and Torboli, 1948; and Mazur et al., 1950), or lysosomal fluid (Coffey and de Duve, 1968).

Treatment with proteinase-K does not digest purified, commercially available preparations of unfenatured horse spleen holo- or apo-ferritin (Atkinson et al., 1989), whereas ferritin subunits are rapidly degraded by proteinase-K (not shown) and by other enzymes (Harrison and Hofmann, 1962). The

resistance of purified holo- and apo-ferritin to these enzymes has led to the development of this method, employing proteinase-K, for rapidly purifying holo- and apo-ferritin from red blood cell lysates. Treatment with proteinase-K has also been successfully used to purify ferritin from homogenates of mouse liver, brain, and spleen, as well as from insect fat body (Nichol and Locke, 1989).

The procedure for isolating ferritin requires only two steps to obtain high yields of pure ferritin, and, depending on its eventual use, may or may not require a Sephadex G200 step (Gonyea et al., 1976) to separate it from any residual proteinase-K used in its purification. The first step involves treating a postmitochondrial supernatant (PMS) with proteinase-K for 15 minutes at 37°C, and the second step involves heat denaturation which inactivates and precipitates the enzyme, as well as any proteinase-K-resistant, heat-labile proteins. Heat denaturation accounts for most of the ferritin lost by this treatment (Atkinson et al., 1989).

Heat denaturation by itself is not sufficient to purify ferritin; the supernatant produced by this treatment contains non-ferritin protein contaminants. These contaminants are not found in samples that have been treated with the enzyme. The absence of any detectable interaction of ferritin antibodies with these other protein bands seen in the stained gels and fluorograms of ferritin preparations not treated with proteinase-K indicates that these proteins are not degradation

products of ferritin or aggregates of ferritin subunits. Instead these additional bands represent other cytoplasmic proteins which are sensitive to proteinase-K but which coisolate with and/or are associated with ferritins prepared by heat denaturation.

Ferritin polymers prepared with proteinase-K are also slightly more mobile in native and SDS gels as compared to ferritin polymers prepared by heat denaturation alone. This results from some non-ferritin protein contaminants which remain bound to electrophoretically-separated ferritin not treated with the enzyme. Proteins that do not react with ferritin antibodies, presumably non-ferritin proteins, are detectable as constituents which remain associated with SDS-PAGE-separated ferritin polymers not treated with proteinase-K. The absence of any contaminating, non-ferritin proteins in ferritin polymers prepared with proteinase-K supports the contention that the slightly greater electrophoretic mobility of the proteinase-K prepared ferritin polymers results from the enzymatic removal of contaminating, non-ferritin proteins. These contaminants may be associated with ferritin *in vivo*, or may associate with it as a result of the heat denaturation treatment. The other possible explanation for the difference in mobility, that there is degradation of ferritin and/or loss of iron from ferritin as a result of the action of proteinase-K, has been shown to be untrue (Atkinson *et al.*, 1989).

It is also not practical to isolate ferritin by proteinase-K treatment alone. The enzyme remains active under very harsh conditions, and even fragments of the enzyme retain proteolytic activity. While it is possible to separate the enzyme from ferritin by gel filtration (Gonyea et al., 1976), one is still faced with the problem of a large amount of active proteinase-K being exposed in the laboratory, and unless the most stringent of precautions are undertaken, it will contaminate other equipment. Thus heat denaturation serves to inactivate the enzyme as well as denature any proteinase-K-resistant proteins.

There has been a recent development that is of interest to this procedure. A column has been developed (MoBiTec; Göttingen, Germany) that has proteinase-K covalently bound to a porous matrix. Heat inactivation of the enzyme is not required because the enzyme does not elute from the column. This column has proven to be very useful for preparations that have been at least partially purified or for purification of small amounts of ferritin (see Chapter 3). It may be practical, in the case of the column, to heat denature the sample first. This would eliminate heat-labile proteins that might be resistant to proteinase-K. Unfortunately, the columns are small, expensive, and have a relatively short life-span and it remains more practical to isolate large quantities of ferritin by the two-step procedure described.

The clear supernatant resulting from heat denaturation of proteinase-K-treated lysate contains ferritin, as shown by electron microscopy and immunochemistry, and is free of all other cellular proteins, as demonstrated by staining and fluorography of samples separated by gel electrophoresis. One- and two-dimensional polyacrylamide gel electrophoretic characterizations of the ferritins isolated in the presence of proteinase-K demonstrate that the ferritin subunits are intact and have relative molecular masses and isoelectric points that are the same as the ferritin subunits isolated in the absence of proteinase-K. Quantitative measurements of ferritin, isolated by this method, indicate that more than 90% of the ferritin is recovered from the PMS of quail red blood cells (Atkinson et al., 1989).

CHAPTER 2 - Ferritin is a heat shock protein of avian reticulocytes

2.1 INTRODUCTION

2.1.1 The significance of the heat shock response

Organisms respond to environmental stresses with immediate and dramatic changes in gene expression. These changes are manifested by an increase in the rate of synthesis of some proteins, and a decrease in the rate of synthesis of others. Changes in the rate of synthesis are regulated typically at both the transcriptional level (by the new and/or enhanced transcription of a small family of genes) and the translational level (by the preferential translation of the mRNA transcripts from these genes).

One particular response to environmental stress that has been studied a great deal is the change in gene expression as a result of rapid elevations in temperature (heat shock) (see reviews in Atkinson and Walden, 1985; Pardue *et al.*, 1988; Morimoto *et al.*, 1990). In fact, heat shock was the first environmental stress recognized to alter gene expression (Ritossa, 1962). The study of the heat shock response has changed gradually from the enumeration of the gene products (the so-called heat shock proteins) to studies of the function

of these proteins and of the factors which regulate their expression.

The significance of the heat shock response is a matter of some debate. For the most part an organism's ability to withstand a heat shock is severely limited. Most organisms can only withstand a heat shock that is short in duration, and which is generally restricted to a relatively small change in temperature (Morimoto et al., 1990). Also, most studies on heat shock have been at the cellular level which makes extrapolation to the whole organism difficult. As a result, the debate continues as to whether heat shock proteins perform specific duties (previously not required) to ensure the survival of the cell (and hence the organism), or whether they are performing the same function, but at an increased level of activity. Much of this debate centers around the definition of heat shock proteins (Morimoto et al., 1990) as those whose enhanced synthesis is the result of an "acceleration in the rate of expression of a small number of specific genes". This definition infers that there is a cellular signal and/or factor that recognizes stress and initiates the cell's response.

2.1.2 The heat shock response of quail reticulocytes

Quail reticulocytes retain the capacity to express a complete heat shock response *in vitro* as well as *in situ* (Atkinson and Dean, 1985; Dean and Atkinson, 1985; Atkinson *et al.*, 1986). These earlier studies revealed that in addition to the new and/or enhanced synthesis of heat shock proteins with relative molecular masses (M_r) of 90,000, 70,000, and 26,000, there was also enhanced synthesis of a protein with an M_r greater than 400,000, and the heat shock-induced enhanced synthesis of this high molecular weight protein appeared to be insensitive to actinomycin D (Atkinson *et al.*, 1986). The possibility that heat shock affected the synthesis of this protein at the translational level only, prompted me to pursue the characterization and identification of this protein. I tentatively identified this protein as the iron storage protein ferritin, and the focus of this aspect of my thesis problem was aimed at confirming the identity of this protein by different criteria and elucidating the mechanism underlying its heat shock-induced enhanced synthesis.

2.2 MATERIALS AND METHODS

2.2.1 Blood collection and radio-labelling of protein

Adult (~3 month-old) Japanese quail (Coturnix coturnix japonica) were made anemic by three successive daily subcutaneous injections of neutralized phenylhydrazine hydrochloride (15 mg/kg). Two days after the third injection, birds were killed by decapitation and blood was collected into 15 ml conical bottom tubes (Falcon 2095; Becton Dickinson, Lincoln Park, NJ) containing 0.1 ml Hepalean (Organon Canada, Toronto, ONT). The red blood cells were enriched for reticulocytes by pelleting them through Ficoll-Paque (Pharmacia, Uppsala, Sweden) by the method of Dean and Atkinson (1985). Washed reticulocytes were resuspended in 75 cm^2 Tissue Culture Flasks (Corning Glass Works, Corning, NY) containing methionine-free medium (1X Minimum Essential Medium, with Earle's Salts and sodium bicarbonate, without methionine, without glutamine; GIBCO, Grand Island, NY) at a concentration of ~0.3-1.2 x 10^6 cells/ml, and incubated at 37°C for 30 minutes. The cells were radioactively labelled by the addition of 60 μCi/ml of L-^{35}S-methionine (Dupont-New England Nuclear, Boston, MA; specific activity >1000 Ci/mmole). Cell cultures were incubated at temperatures ranging from 37 to 46°C for 2 hours with agitation of the flasks at 30 minute intervals.

2.2.2 Production of a postnuclear supernatant

Labelled cells were collected in 50 ml conical bottom tubes (Falcon 2098; Becton Dickinson) and pelleted at 100 x g for 10 minutes. The cells were washed in 5 volumes of methionine-free medium and repelleted at 100 x g for 10 minutes. Pelleted cells were lysed in 4 volumes of a buffer (10 mM Tris-HCl (pH 7.2), 150 mM NaCl, 5 mM $MgCl_2$, 2 mM EGTA, 0.25 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) containing 1% Triton X-100 (Blikstad and Lazarides, 1983) and left on ice for 30 minutes. The lysate was centrifuged at 200 x g for 10 minutes, and the postnuclear supernatant (PNS) was stored, either briefly at 4°C, or frozen at -70°C, prior to use.

2.2.3 Heat denaturation treatment

Aliquots of samples were placed in capped 1.5 ml Eppendorf centrifuge tubes and heat-denatured by placing them at 75°C for 15 minutes. The heat-denatured samples were placed on ice for 30 minutes, centrifuged at 5,000 x g for 5 minutes, and the supernatants (containing nondenatured protein) were transferred to separate tubes for storage (at 4°C) and analysis. If necessary, samples were concentrated using Amicon Centricon 30 Microconcentrators (Amicon Corp. Danvers, MA) prior to storage.

2.2.4 Proteinase-K treatment

Proteinase-K, prepared in 80 mM Tris-HCl (pH 6.8), was added to the PNS so that the final concentration of enzyme was 0.2 μg/μl (i.e. 1 μg of proteinase-K/80 μg of protein). The samples were incubated in capped tubes at 37°C for 15 minutes on a shaker bath. After the 37°C incubation, the samples were heated at 75-80°C for 15 minutes to inactivate and precipitate the denatured enzyme, and subsequently cooled on ice for 30 minutes. The cooled samples were centrifuged and the resulting clear supernatants were either transferred to separate tubes for storage or concentrated in type CF50A Centriflo membrane cones and stored at 4°C or -70°C.

2.2.5 Measurement of radioactivity

Radioactively-labelled protein in aliquots of control and heat-shocked PNS was precipitated by the addition of 1/3 volume 50% trichloroacetic acid (w/v). Samples were mixed gently, placed on ice for 30 minutes, and centrifuged (5,000 x g, 10 minutes). The pellets were rinsed with diethylether, and recentrifuged. The ether was removed, and the pellets were dried *in vacuo* overnight. The dried pellets were resuspended in 80 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-MEt, and aliquots were removed for liquid scintillation counting.

Radioactivity was measured by a Beckman LS-255 liquid scintillation counter (Beckman Instruments, Palo Alto, CA) using a Triton X-100 - toluene - Omnifluor (Dupont-New England Nuclear, Dorval, Québec) cocktail.

2.2.6 Polyacrylamide gel electrophoresis

2.2.6.1 One dimensional SDS-PAGE

One-dimensional sodium dodecyl sulfate (SDS) polyacrylamide slab gels consisted of either a 3-15% or a 7.5-17.5% separating gel (0.375M Tris-HCl (pH 8.9), 0.1% (w/v) SDS) overlaid with a 3% stacking gel (0.125M Tris-HCl (pH 6.8), 0.1% (w/v) SDS. Running buffer contained 25 mM Tris-HCl (pH 8.3), 192 mM glycine, 0.1% (w/v) SDS. Samples were made to contain 80 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol (β-MEt), 10% (v/v) glycerol, and 0.1% Bromphenol Blue. Samples were clarified by centrifugation in a Beckman microfuge (Beckman Instruments, Spinco Div., Palo Alto, CA) prior to electrophoresis.

Holo- and apo-ferritins were separated on linear gradient gels of 3-15% polyacrylamide, whereas ferritin subunits, generated by boiling the samples for 3 minutes in 2% SDS and 5% β-mercaptoethanol (Schaefer and Theil 1981), were separated on linear gradient gels of 7.5-17.5% acrylamide.

Electrophoresis was done at 5 mA/gel until the dye front had moved into the separating gel, and then at 15 mA/gel until the dye front had run off. Gels were stained in 0.1% (w/v) Coomassie Blue R (C.I. 42660; Sigma Chemical Co., St. Louis, MO) in methanol:water:acetic acid (40:50:10), destained in methanol:water:acetic acid (10:80:10), and stored in water:acetic acid (90:10).

The relative molecular mass was estimated by comparing their distance of migration through the separating gel to a standard curve of Log(Molecular weight) (ordinate) vs Distance Migrated (abscissa) prepared using marker proteins of known molecular weight (Low Molecular Weight Calibration Kit: phosphorylase b - 94,000 Da; bovine serum albumin - 67,000 Da; ovalbumin - 43,000 Da; carbonic anhydrase - 30,000 Da; soybean trypsin inhibitor - 20,000 Da; α-lactalbumin - 14,400 Da; Pharmacia/LKB) separated on the same gel, as well as ferritin and apoferritin (Sigma).

2.2.6.2 Two-dimensional separations

Two-dimensional separation of proteins was done according to the method of O'Farrell (1975) with some minor modifications (see below). Separation in the first dimension was achieved by isoelectric focussing (i.e. proteins were separated on the basis of their pI); and in the second dimension was achieved by SDS-PAGE (i.e. separated on the basis of size).

The isoelectric focussing gel was a 3% polyacrylamide tube gel containing 9M urea, 2% NP-40, and 2% ampholines (Pharmacia/LKB, Bromma, Sweden; pH 3.5 to 10). These gels were pre-run (to establish the pH gradient) at 200V for 15 minutes, 300V for 30 minutes, and 400V for 30 minutes. The upper buffer was 0.25N NaOH, and the lower buffer was 0.2N H_3PO_4. The lower buffer was cooled by a water jacket to ~10°C. After the pre-run, the upper buffer was removed from the chamber and from the tops of the tube gels, and the samples (made to contain 9M urea, 1% NP-40, 2% ampholines, and 5% β-MEt) were applied. Overlay buffer (4.5M urea, 1% ampholines) was applied, and covered with fresh upper buffer. Gels were electrophoresed at 400V for ~18 hrs, and then at 800V for 2 hrs. After electrophoresis, tube gels were extruded into a solution containing 80 mM Tris-HCl (pH 6.8), 2% SDS, and 5% β-MEt, and allowed to equilibrate for 30 minutes (with agitation). The tube gels were adhered to the top of slab gels (either a 3-15% or a 7.5-17.5% SDS-polyacrylamide separating gel with a 3% stacking gel) with 1% agarose (containing 80 mM Tris (pH 6.8), and 0.1% SDS) and run as previously described.

2.2.7 Fluorography of polyacrylamide gels

Destained gels were soaked in three changes of dimethylsulfoxide (DMSO) to remove all traces of water and acetic acid. Each gel was then impregnated with 2,5-diphenyloxazole

(PPO) by soaking in a 22% (w/v) solution of PPO in DMSO. The PPO-impregnated gels were immersed in a vast excess of water to precipitate the PPO, and dried down on to Whatman 3MM filter paper using a Bio-Rad Model 224 Gel Dryer (Bio-Rad Laboratories, Richmond, CA). The dried gels were taped to stiff cardboard and inserted into cassettes apposed to a piece of Kodak X-Omat RP X-ray film (Eastman Kodak, Rochester, NY) preexposed to an absorbance (540 nm) of ~0.15 (Laskey and Mills, 1975). Exposure of the film was carried out at -70°C.

2.2.8 Immunoblotting analysis of the ~450 kDa protein

Electrophoretically-separated proteins were transferred (using a Bio-Rad TransBlot Cell (Bio-Rad Laboratories)) at 60V for 8 hours to nitrocellulose (DuPont-New England Nuclear, Boston, MA) using 25 mM Tris (pH 8.3), 192 mM glycine, 20% v/v methanol as the transfer buffer (Towbin et al., 1979). The nitrocellulose was blocked for 1 hour (at room temperature) in a solution of 3% (w/v) gelatin (Bio-Rad Laboratories) in tris-buffered saline (TBS: 20 mM Tris-HCl (pH 7.5), 500 mM NaCl). The blocked nitrocellulose was incubated in primary antibody solution containing rabbit anti-horse ferritin (shown to cross-react with avian ferritins; Theil and Brenner (1981)) (Cappel Research Products; Organon Teknika, Scarborough, ONT) diluted 1:3,000 (in TBS containing 1% (w/v) gelatin) for 8 hours at room temperature, washed twice (5 minutes each) with

TBS, and incubated in secondary antibody solution containing goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad Laboratories) diluted 1:1,000 (in TBS) for 2 hours at room temperature. Following the incubation with the secondary antibody the nitrocellulose was washed twice (5 minutes each) with TBS.

Cross-reaction of primary and secondary antibodies was visualized using 4-chloro-1-naphthol (4-CN; Bio-Rad Laboratories) as a substrate for the horseradish peroxidase conjugated to the secondary antibody. A solution of 60 mg of 4-CN in 20 ml of methanol was added to a solution of 100 ml TBS containing 60 μl 30% H_2O_2, immediately prior to use. The washed membranes were incubated in the resulting solution for 30 minutes (in the dark), and colour development was stopped by rinsing in distilled water. The developed immunoblots were stored in water (in the dark) until photographed.

2.2.9 Staining of polyacrylamide gels for the presence of iron

Gels were stained for iron in a solution of 2% acetic acid, 0.2% mercaptoacetic acid, and 0.75mM sodium ferene made up in glass distilled water (Chung, 1985). Gels were photographed, stained for protein with Coomassie brilliant blue G, photographed, and prepared for fluorography (when applicable).

2.2.10 Determination of the specific activity of ferritin synthesized by control and heat-shocked reticulocytes

Control and heat-shocked ferritins were isolated by treatment with proteinase-K, or by electroelution from excised regions of polyacrylamide gels (located by fluorography, and by immersion of gels in cold 0.25M KCl (Hager and Burgess, 1980). The protein concentration of these samples was measured by the method of Comings and Tack (1972) and the amount of radioactivity (as trichloroacetic acid-precipitable cpm) was measured. Specific activity was expressed as cpm/μg protein.

2.2.11 Electron microscopy

Small drops (0.5 μl) of an aqueous solution of electrophoretically purified ~450 kDa protein or horse spleen ferritin (Sigma) were smeared across Formvar-coated, carbon-shadowed copper grids with a piece of filter paper and allowed to air dry. Unstained preparations were viewed directly. Other preparations were negatively stained with sodium phosphotungstate (Locke and Huie, 1980) or were stained with bismuth at pH 9 by the method of Nichol and Locke (1989). Grids were viewed on a Philips EM 200 at 80 kV.

2.3 RESULTS

2.3.1 One-dimensional SDS-PAGE analysis of L-[^{35}S]-methionine-labelled proteins synthesized by quail reticulocytes

Quail reticulocytes subjected to brief elevations in temperature exhibit the new and/or enhanced synthesis of polypeptides with M_r values of approximately 26,000, 70,000, 90,000, and ~450,000 (Figure 2.1). Although the M_r ~450,000 polypeptide is constitutively expressed in reticulocytes, its synthesis appears to be enhanced in lysates from reticulocytes incubated for 2 hours at temperatures greater than 40°C (Figure 2.1A). The electrophoretic mobility of this ~450 kDa protein appears to change when the samples are boiled for 2 minutes in the presence of SDS and β-mercaptoethanol; after this treatment the ~450 kDa protein becomes less apparent, and there is a concomitant increase of a polypeptide of approximately 21 kDa (Figure 2.1B).

2.3.2 Characterization of the ~450 kDa Hsp as ferritin

Analysis of the proteins separated by one-dimensional SDS-PAGE from lysates of control (not shown) and heat-shocked cells reveals that the ~450 kDa protein contains iron (Figure 2.2A, Lane 2), crossreacts with antibodies to horse spleen ferritin (Figure 2.2A, Lane 3), is heat stable

Figure 2.1. One-dimensional SDS-PAGE analysis of L-[^{35}S]-methionine labelled proteins synthesized by quail reticulocytes. These samples were obtained from low speed supernatants of Triton X-100 lysates, and contain equal amounts of protein (80 μg). Panel A, fluorograms of SDS-PAGE-separated proteins synthesized by quail reticulocytes incubated for 2 hours at 37°, 40°, 42°, 44°, and 46°. Samples were made to contain 2% SDS, and were applied directly to gels. Cells maintained for 2 hours at temperatures above 40°C show enhanced synthesis of a protein with a molecular mass of ~450,000 as well as the previously reported 26, 70, and 90 kDa Hsps (see arrowheads; Atkinson and Dean, 1985). Panel B, fluorograms of SDS-PAGE-separated proteins synthesized by quail reticulocytes incubated for 2 hours at 37° (control; C) or 45°C (heat shock; HS). Samples were made to contain 2% SDS, and either applied directly to gels (i.e. not boiled, (-Δ), C and HS lanes on the left-hand side of panel B) or boiled prior to electrophoresis (+Δ, C and HS lanes on the right-hand side of panel B). There is a decrease in abundance of the newly synthesized ~450 kDa protein and a concomitant increase in a 21 kDa protein, suggesting that boiling in the presence of SDS and β-mercaptoethanol separates the ~450 kDa protein into 21 kDa subunits. Marker proteins of relative molecular mass (M_r x 10^{-3}) are indicated.

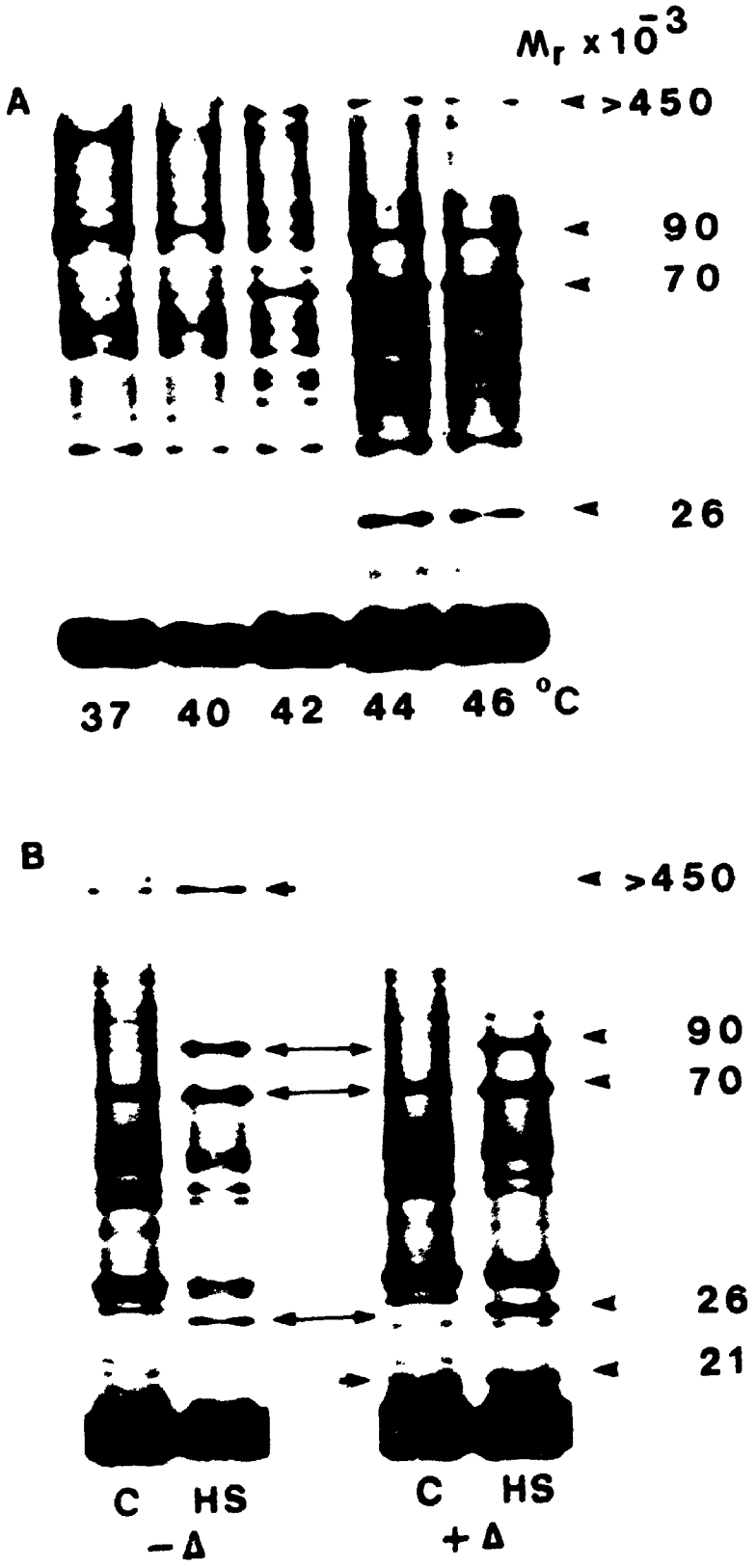
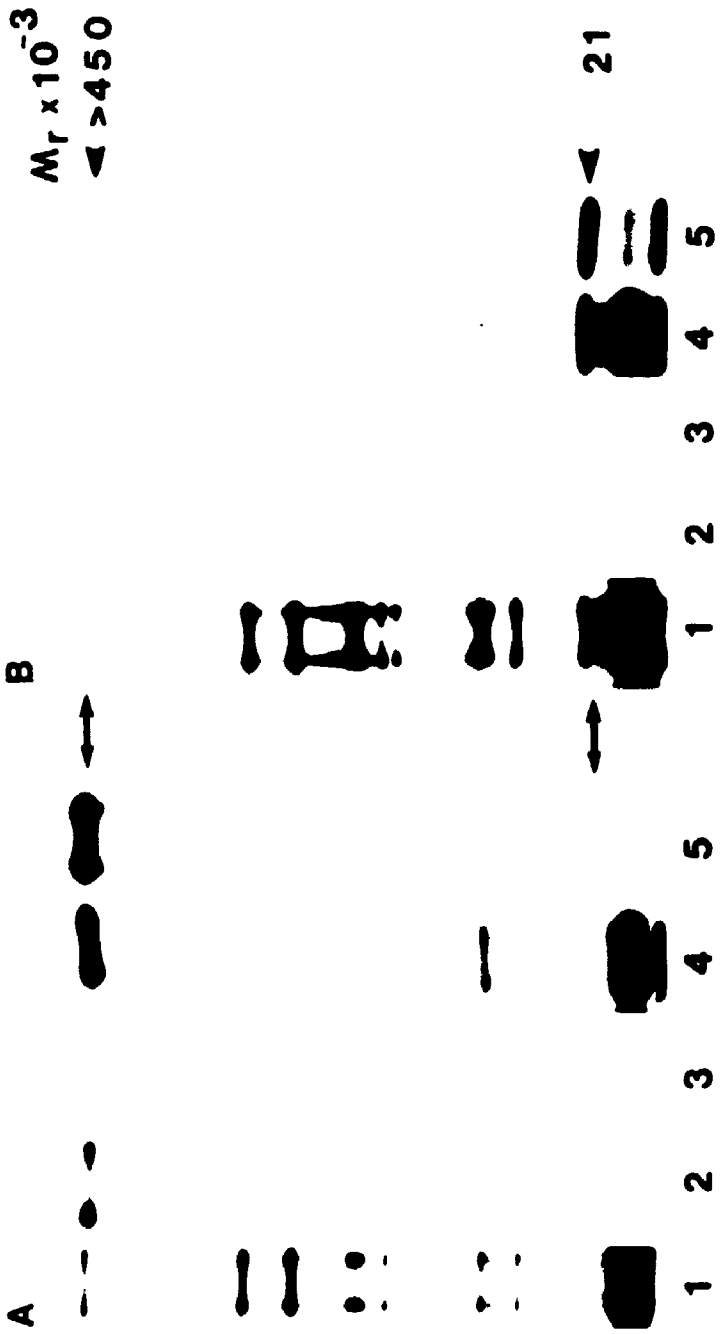

$M_r \times 10^{-3}$
A
>450
90
70
26
37 40 42 44 46 °C
B
>450
90
70
26
21
C HS
−Δ
C HS
+Δ

Figure 2.2. One-dimensional SDS-PAGE separation of quail reticulocyte PMS, and subsequent analyses. Samples are from heat-shocked reticulocytes which were lysed in Triton X-100 and made to contain 2% SDS. Equal amounts of protein (80 μg) were applied to Lanes 1-3, and equal volumes of 10x concentrated supernatants were applied to lanes 4 and 5. Lanes 1, 4, and 5 are fluorograms. Lane 1, heat shock PMS; lane 2, the gel was stained for the presence of iron by the method of Chung (1985); lane 3, the electrophoretically-separated proteins were transferred to nitrocellulose and incubated with antibodies to horse spleen ferritin; lane 4, the sample was heat-denatured prior to electrophoresis; lane 5, the sample was treated with proteinase-K. Panel A, samples were applied directly to gels. The ~450 kDa protein, like ferritin, contains iron and immunoreacts with ferritin antibodies. It remains in the supernatant of heat-denatured samples and is resistant to proteinase-K. Panel B, samples were adjusted to contain 2% SDS and boiled prior to electrophoresis. The ~450 kDa protein separates into 21 kDa subunits which react with ferritin antibodies but, like ferritin, no longer contain iron. The ~450 kDa protein from lysates of heat-shocked quail reticulocytes has biochemical properties identical to ferritin and, like ferritin, consists of 21 kDa subunits. The position of the ~450 kDa protein and its 21 kDa subunit are indicated.

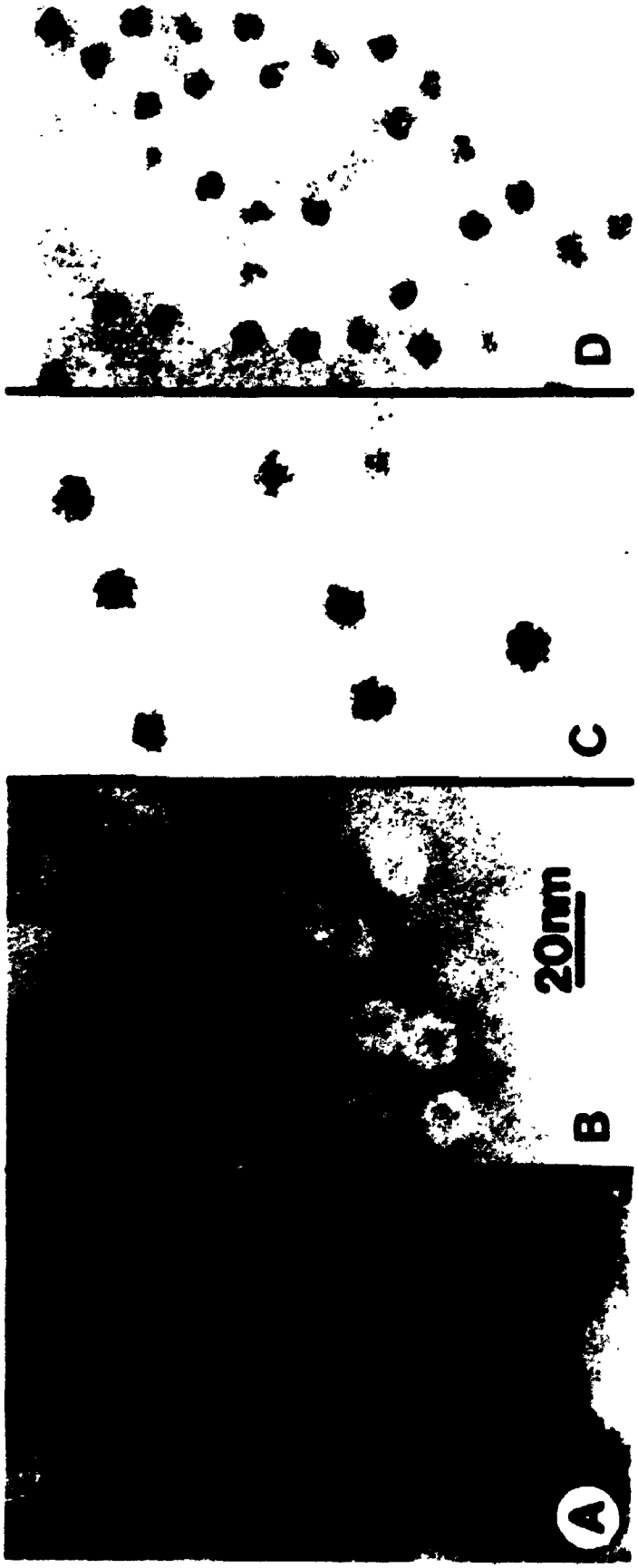

$M_r \times 10^{-3}$
>450
21
A
B
1
2
3
4
5
1
2
3
4
5

(Figure 2.2A, Lane 4), and is resistant to proteinase-K treatment (Figure 2.2A, Lane 5). Separation of lysates of control (not shown) and heat-shocked cells by one-dimensional SDS-PAGE where the samples are boiled in the presence of SDS and β-mercaptoethanol prior to electrophoresis reveals that the ~450 kDa native protein is separated into polypeptides of ~21 kDa that do not contain iron (Figure 2.2B, Lane 2), but which do crossreact with antibodies to horse ferritin (Figure 2.2B, lane 3). The heat-resistant and proteinase-K resistant M_r ~450,000 protein isolated from control (not shown) and heat-shocked cells (Figure 2.2A, lanes 4 and 5) also still separates into 21 kDa subunits (Figure 2.2B, lanes 4 and 5).

2.3.3 Electron microscopic examination of purified ~450 kDa protein

The ~450 kDa protein (electroeluted from excised gel slices of electrophoretically-separated proteins) is indistinguishable from horse spleen ferritin (Figure 2.3, Panels A and B). Also: 1) bismuth-stained preparations of the ~450 kDa particles (Figure 2.3C) reveal a central core with axial projections, and 2) unstained preparations (Figure 2.3D) show an electron dense (iron) core, ~7 nm in diameter.

Figure 2.3. Electron microscopy. Panel A, negatively stained horse spleen ferritin. Panel B, negatively stained >450 kDa protein. Panel C, bismuth stained >450 kDa protein. Panel D, unstained >450 kDa protein. The ~450 kDa protein from lysates of heat-shocked quail reticulocytes is similar in size and appearance to horse spleen ferritin. It has a central core with axial projections, and it has an electron dense (iron) core. Scale, bar = 20 nm.


A
B
20nm
C
D

2.3.4 One- and two-dimensional PAGE comparisons of the electrophoretic mobilities of ferritin subunits from control and heat-shocked cells

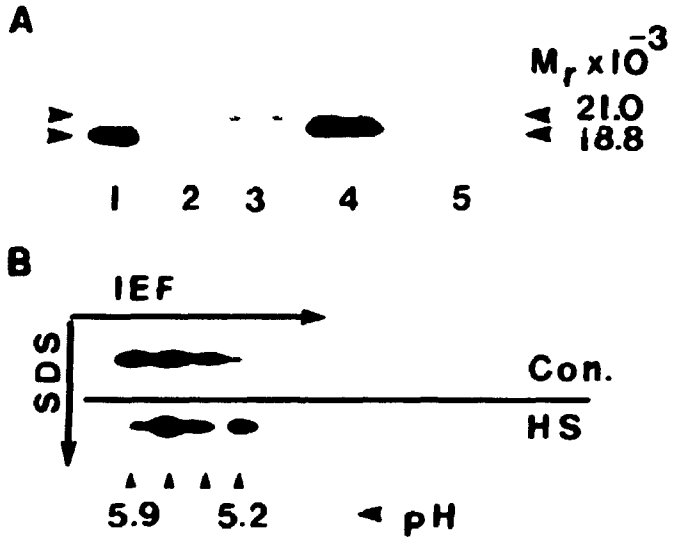
Separation of ferritin subunits by one-dimensional SDS-PAGE reveals that the ferritin subunits synthesized during heat shock have a very similar, if not identical, mobility to the ferritin subunits synthesized under control conditions, with an M_r ~21,000 (Figure 2.4A). The mobility of the quail ferritin subunits is slightly less than that of horse spleen ferritin subunits (Figure 2.4A, lanes 1 and 4), and very similar to that of subunits of human heart ferritin (Figure 2.4A, lane 5).

Separation of ferritin subunits by two-dimensional IEF-SDS-PAGE also reveals that the ferritin subunits synthesized during heat shock are very similar to those synthesized under control conditions, with pI values of 5.9, 5.5, 5.3, and 5.2 (Figure 2.4B).

2.3.5 Comparison of the specific activity of ferritin and ferritin subunits synthesized during control or heat shock

Samples from the low speed supernatant of the control and heat shock lysates were treated with proteinase-K, heat-denatured, and centrifuged (see Chapter 1 and Atkinson *et al.*,1989). The supernatants, containing pure ferritin

Figure 2.4. One- and two-dimensional PAGE comparisons of the mobility of ferritin subunits from control and heat shock reticulocytes. Samples were made to contain 2% SDS and boiled prior to electrophoresis. Panel A, Coomassie-blue-stained one-dimensional gels. Lanes 1 and 4, subunits of horse spleen ferritin; lane 2, ferritin subunits from control (2 hours at 37°C) reticulocytes; lane 3, ferritin subunits from heat-shocked (2 hours at 45°C) reticulocytes; lane 5, subunits of human heart ferritin. The subunits of ferritin from control and heat-shocked avian reticulocytes have the same M_r, and are similar in mobility to the subunits of horse spleen ferritin and human heart ferritin. Panel B, fluorograms of two-dimensional PAGE-separated ferritin subunits from Control (C) and heat-shocked (HS) quail reticulocytes. N.B. Only the ~21 kDa region of the second dimension gel is shown. Isoelectric points were estimated from a standard curve obtained from a sample-free isoelectric focussing gel that was run at the same time. The subunits of ferritin from control and heat-shocked avian reticulocytes have similar pIs as well as the same M_r.


A
$M_r \times 10^{-3}$
21.0
18.8
1 2 3 4 5
B
IEF
SDS
Con.
HS
5.9 5.2
pH

polymers, were assayed for protein concentration by the method of Comings and Tack (1972) and radioactivity was measured by liquid scintillation counting. Samples of electrophoretically-separated control and heat-shocked ferritins were also obtained from regions of polyacrylamide gels located by fluorography, or by immersion of gels in cold 0.25M KCl (Hager and Burgess, 1980), and were analyzed for protein concentration and radioactivity. The specific activity of ferritin increases from ~90 cpm/μg in control, to 250-440 cpm/μg in heat-shocked cells (Table 2.1).

Table 2.1. Summary of the specific activity (cpm/μg) of ferritin isolated from control and heat-shocked reticulocytes. Determination of the specific activity of ferritin purified from control and heat shock PNS indicates that while heat shock reduces the incorporation of radioactivity for the entire lysate to ~60% of control levels (not shown), it stimulates the synthesis of ferritin ~3-5 times (from ~90 cpm/μg of ferritin in control to ~250-440 cpm/μg of ferritin in heat-shocked cells). Cells were labelled from t=0 until t=2 hours, or from t=2 to t=4 hours.

	Specific activity (cpm/μg protein)		
Duration of Heat Shock	Control	Heat shock	Increase
2 hours	87	248	~2.8x
4 hours	91	439	~4.8x

2.4 DISCUSSION

Quail reticulocytes exhibit the heat shock-induced increase in the synthesis of a ~450 kDa protein that is constitutively expressed. This protein possesses several properties that are peculiar to ferritin. Native ferritin (M_r ~450,000) is normally heat stable (i.e. it is not precipitated or separated into its subunits by a 10 minute incubation at 75-80°C in aqueous solution at pH 7.0 (Hofmann and Harrison, 1963; Listowsky et al., 1972)), is insensitive to a number of proteolytic enzymes including proteinase-K (Coffey and de Duve, 1968; Atkinson et al., 1989; Nichol and Locke, 1989), generally contains iron (i.e. holo-ferritin; Theil, 1987), displays a characteristic appearance in the electron microscope (Munro and Linder, 1978; Nichol and Locke, 1989), and can be separated into its ~21 kDa subunits by boiling in the presence of SDS and β-mercaptoethanol (Schaefer and Theil, 1981; Atkinson et al., 1989). The ~450 kDa protein separates into 21 kDa subunits when the sample is boiled in the presence of SDS and β-mercaptoethanol prior to electrophoresis. It contains iron; remains in the supernatant of heat-denatured samples; is resistant to proteinase-K treatment; and both the ~450 kDa polymer and its 21 kDa subunits crossreact with antibodies to horse spleen ferritin. The ~450 kDa protein is indistinguishable from horse spleen ferritin when examined by electron microscopy, and exhibits other morphological features

characteristic of ferritin including: axial projections of the core as revealed by bismuth-staining (Locke and Huie, 1980), and an electron dense (iron) core.

Confirmation of the actual enhancement of ferritin synthesis was obtained by measuring the specific activity (cpm/μg) of the ferritin polymers isolated from control and heat-shocked cells. The results indicate that even though heat shock reduces the incorporation of radioactivity to ~60% that of control, there is a 3- to 5-fold increase in the rate of synthesis of ferritin.

Ferritin polymers (M_r ~450,000) can be composed of subunits having slightly different M_r and pI values in different cells and, under different physiological conditions, in the same cell (Theil, 1987; Mertz and Theil, 1983), thus the M_r and pI values of the ferritin subunits synthesized under control or heat shock conditions were compared. Ferritin synthesized in response to heat shock appears to be made up of subunits that are virtually identical to those synthesized under control conditions, i.e. quail ferritin appears to consist entirely of H(eavy) ~21 kDa subunits.

Ferritins are iron sequestering and iron storage proteins found in virtually all cells (Theil, 1987). Iron administration to animals, plants, or cells in culture stimulates the synthesis of ferritin subunits (Theil, 1987; Munro and Linder, 1978). The iron-induced enhanced synthesis of ferritin is primarily regulated at the post-transcriptional level and involves a mobilization of a cytoplasmic pool of free, inactive ferritin mRNAs on to polyribosomes (Zahringer et al., 1976; Aziz and Munro, 1986). The translational regulatory machinery involved in the iron-induced activation of these mRNAs includes an iron-responsive element of 28 highly conserved nucleotides in the 5'-untranslated region of ferritin mRNAs (Hentze et al., 1987; Aziz and Munro, 1987; Stevens et al., 1987), and a ferritin repressor protein which inactivates ferritin mRNAs by binding to the iron-responsive element (Leibold and Munro, 1988; Rouault et al., 1988; Walden et al., 1988). Increased intracellular levels of chelatable iron (Rogers and Munro, 1987; Mattia et al., 1989) or other forms of iron, such as hemin (Lin et al., 1990), have been postulated to interact with the ferritin repressor protein, either directly or indirectly, and mediate the derepression of ferritin mRNAs.

The heat shock induction of ferritin synthesis in reticulocytes is similar to that established for the iron induction of ferritin synthesis in a number of cell types in that its enhanced synthesis appears to be partially, if not

wholly, regulated at the post-transcriptional level. The heat shock-induced synthesis of ferritin is insensitive to actinomycin D and appears to involve a translocation of H-ferritin mRNAs from a polyribosome-free, translationally repressed state to a polyribosome-associated, translationally active state (Atkinson et al., 1990). Unlike heat shock and iron, which appear to act by enhancing the translation of ferritin mRNA, an unidentified factor present in reticulocyte lysates, but not in wheat germ lysates, represses the translation of ferritin mRNA (Dickey et al., 1988). Other agents, such as tumor necrosis factor-α (Torti et al., 1988) and thyrotropin (Cox et al., 1988), appear to regulate ferritin synthesis by enhancing the transcription of ferritin mRNA.

The actual mechanism by which heat shock (stress) affects the induction of specific transcriptional and translational changes in cells is not known. However, results from a number of studies support the hypothesis that heat shock initiates the generation of denatured and abnormal proteins which are proposed to mediate, directly or indirectly, the new and/or enhanced expression of the heat shock proteins (Kelley and Schlesinger, 1978; Hightower, 1980; Kelley and Schlesinger, 1982; Goff and Goldberg, 1985; Munro and Pelham, 1985; Ananthan et al., 1986; Edington et al., 1989). Regardless of what the case may be for affecting a general heat shock response, it has been demonstrated that the enhanced synthesis of ferritin detected in heat-shocked reticulocytes is a direct

result of the derepression and translocation of ferritin mRNAs (Atkinson et al., 1990). The mechanism by which inactive, free cytoplasmic ferritin mRNAs are derepressed most likely involves the destabilization or removal of the ferritin repressor protein from the iron-responsive elements (Leibold and Munro, 1988; Rouault et al., 1988; Walden et al., 1988). Thus, in keeping with the abnormal and/or denatured protein hypothesis mentioned above, Atkinson et al. (1990) have proposed that heat stress affects the induction of enhanced ferritin synthesis by denaturing (or destabilizing) the ferritin repressor protein or by denaturing some intracellular iron-containing proteins, such as hemoglobin. In the latter case, the denaturation of hemoglobin would lead to the release of iron or hemin-like products into the intracellular environment which, in turn, could destabilize or remove the ferritin repressor protein, derepress the ferritin mRNAs, and promote ferritin synthesis.

Heat shock proteins were thought originally to provide protection for cells by allowing them to survive high temperatures, i.e. these proteins would perform functions not normally required by the cell. However, the fact that heat shock proteins were constitutively expressed in the cell suggested that they were required for normal growth and development, and that their increased synthesis was in response to an increased requirement for whatever function they normally provided (Morimoto et al., 1990). This idea is

supported by the occurrence of ferritin as a heat shock protein. Although the heat shock response occurs very rapidly, the enhanced synthesis of a heat shock protein that does not require new transcription suggests that this particular response occurs even more immediately, and that it might therefore be a response to a sudden and dramatic change in the intracellular environment, and, as such, provide a protective function essential to the cell's survival. The established and important intracellular function that ferritin normally plays in protecting a cell from the adverse affects of free iron (Munro and Linder, 1978; Theil, 1987), coupled with its enhanced synthesis during heat shock, suggests that there is an essential requirement for additional ferritin in red blood cells enduring thermal stress.

Heat-shocked quail reticulocytes exhibit the new and/or enhanced synthesis of four proteins with M_rs of 26,000, 70,000, 90,000 and >400,000 (Atkinson et al., 1986). The results presented here confirm (by biochemical, immunochemical, and morphological criteria) the identification of the so-called M_r >400,000 protein as the ~450 kDa iron-sequestering protein ferritin, and form the basis of results described in Atkinson et al. (1990) that describe ferritin as a translationally regulated heat shock protein of quail reticulocytes. Although the control of ferritin synthesis by iron has been well documented (Theil, 1987; Munro and Linder, 1978), there

have been few observations on other agents that may affect the expression of ferritin genes (Cox et al., 1988; Dickey et al., 1988; Torti et al., 1988;) and only one other report on the occurrence of ferritin as a heat shock protein (Rimland et al., 1988).

CHAPTER 3 - The "prosome" fraction of avian reticulocytes contains two morphologically and biochemically distinct protein particles: the proteasome and ferritin.

3.1 INTRODUCTION

The "prosome" has been described as a "round", "raspberry-shaped" RNP particle that is found in avian reticulocytes in association with a 20S globin mRNP (Schmid et al., 1984). It also has been studied in cells from such diverse organisms as Drosophila, duck, HeLa cells, mouse, sea urchin, tobacco, and yeast (see reviews in: Martins de Sa et al., 1986; Scherrer, 1990). However, two reports (Falkenburg et al., 1988; Arrigo et al., 1988), citing the similarities in size and subunit composition (among other characteristics), concluded that the prosome is identical to the proteasome, a large, cylindrical multifunctional protease complex (see review in: Rivett, 1989a). I have found that the fraction reported to contain prosomes (isolated by sucrose density gradient ultracentrifugation) actually consists of two morphologically and biochemically distinct particles: one is the proteasome, the other is ferritin. I show that the mobility of ferritin in nondenaturing gels is affected by agents that disrupt RNA-protein interactions, and I propose that ferritin is the component of the 20S globin mRNP (Gander et al., 1973)

that is associated with globin mRNA. I have also demonstrated that the prosome particle is formed by degradation of the proteasome, and I have found that ferritin is the core protein component of the so-called 35S prosome-like-particle.

3.1.1 The prosome

The prosome has its roots in a proposal by Spirin (1964, 1969) who suggested that cytoplasmic mRNAs might exist in a "masked" form in ribonucleoprotein particles ("informosomes"). Several groups (Perry and Kelley, 1968; Henshaw, 1968; Spohr et al., 1970; Morel et al., 1971) were able to confirm Spirin's hypothesis. Spohr and others used avian erythroblasts as a system to study the phenomenon because of the fact that 80 to 90% of the protein synthesized in these cells is hemoglobin (Scherrer and Marcaud, 1968). They showed that polyribosome-associated globin mRNAs were affiliated with specific proteins in a ribonucleoprotein complex (Spohr et al., 1970; Spohr et al., 1972a). This globin mRNP complex was described as a 15S particle (Morel et al., 1973) that consisted of two major protein components of 49 and 73 kDa, and six minor components of 50, 60, 64, 84, 105, and 120 kDa. Morel et al. (1973) and Dubochet et al. (1973) were the first to describe the morphological structure of the duck globin mRNP complex.

Spohr et al. (1972a) reported that ribosome-free cytoplasmic globin mRNP could be isolated from the postribosomal supernatant of duck erythroblasts by sucrose density gradient ultracentrifugation, and demonstrated that the mRNA extracted from these free cytoplasmic mRNPs translated into globin (Spohr et al., 1972b). The free cytoplasmic globin mRNP was characterized by Gander et al. (1973) as a 20S particle, containing a group of proteins from 15-24 kDa, and a 9S RNA that directed the synthesis of globin chains. They proposed that the globin mRNA found in the 20S particle was associated with proteins other than those associated with message engaged in the process of translation, and that the mRNA-associated protein populations were exchanged when the mRNA became functional.

Civelli et al. (1976) showed that while 15S ribosome-associated mRNP, and the 9S RNA extracted from either the 15S ribosome-associated mRNP or the 20S free mRNP could be translated in vitro, the intact, free 20S mRNP did not stimulate cell-free globin synthesis. This inability to translate the globin mRNA when it is associated with the 20S mRNP was taken as direct evidence for the existence of "masked" mRNA as proposed by Spirin (1969). They also showed that this translational repression was not eliminated by high-salt wash treatment, and they concluded that the repressor must be contained within the salt-resistant globin mRNP-core particle. They also found that high salt treatment separated

the 20S mRNP into two major components of 13S and 4S, and reported that the 9S mRNA sedimented with the 13S component.

In 1977, Vincent et al. reported that specific mRNAs were associated with specific proteins in untranslated cytoplasmic mRNP complexes of duck erythroblasts. They reported that the 20S mRNP contained proteins that ranged in size from 20-52 kDa, the major component of which was 48 kDa (as opposed to the 15-24 kDa subunits reported previously (Gander et al., 1973)).

The 20S mRNP was found to dissociate into four subcomplexes of 19S, 16S, 13S, and 4S upon treatment with high-salt (Civelli et al., 1980). The 16S and 13S subcomplexes contained the 9S globin mRNA while the 19S subcomplex consisted almost entirely of protein. Neither the 16S nor the 13S complexes supported the synthesis of globin, and so the authors concluded that the translational repressor must be tightly linked to the globin mRNA.

Vincent et al. (1980) reported that purification of the 20S globin mRNP yielded a single component. This component consisted of 30 major polypeptides ranging from 20-150 kDa, including the 20-52 kDa proteins reported previously (Vincent et al., 1977). The 19S particle obtained from a high-salt wash of the 20S mRNP contained a group of polypeptides ranging in size from 22-30 kDa, and an additional polypeptide of 52 kDa. The 19S particle was reported to contain no globin mRNA. They also separated the 20S fraction (not the high-salt-

treated 20S fraction) on 2.5-6% polyacrylamide tube gels and showed, by hybridization against the RNAs eluted from slices of these gels, that most of the globin mRNA was found with a component or components other than the component consisting of the characteristic 20-52 kDa proteins.

The 19S RNP was named the "prosome" in 1984. It was described as a novel RNP that also contained two small cytoplasmic RNAs of 70-90 nucleotides, but no mRNA (Schmid et al., 1984). The particle was described as being "round" and "raspberry-shaped" with a tendency to stack and form cylindrical particles. This particle was also described as being capable of forming an association with a specific inactive cytoplasmic mRNP (i.e. the 20S globin mRNP described by Garder et al. (1973)).

Since 1984 there have been numerous studies conducted on different aspects of prosomes. Among these are reports on the cytolocalization of prosomes, the glycosylation of prosome proteins, and the labelling of prosome proteins of cultured Drosophila KC cells with ^{35}S-methionine (Grossi de Sa et al. (1988), Gautier et al. (1988), Pal et al. (1988), and Martins de Sa et al. (1989)).

3.1.2 The proteasome

The proteasome is a widely distributed, high molecular weight (~700 kDa) nonlysosomal proteinase (for reviews see:

Rivett, 1989a,b). It consists of ~10-15 protein subunits (ranging in size from ~21--35 kDa) that are assembled into a cylindrical structure.

The characterization of the proteasome began with the observation by Goldberg and St. John (1976) that inhibitors of energy production could reversibly block the selective degradation of abnormal proteins. They showed that there was a correlation between the generation of ATP and the rate of protein breakdown in E. coli (Olden and Goldberg, 1978; St. John and Goldberg, 1978), and that the ATP-stimulated degradation of abnormal proteins involved nonlysosomal cytoplasmic proteases (Etlinger and Goldberg, 1977; DeMartino and Goldberg, 1979; Murakami et al., 1979). DeMartino and Goldberg (1979) and Boches and Goldberg (1980) succeeded in partially purifying the proteolytic activity from 100,000 x g supernatants of rat liver and rabbit reticulocyte lysates, respectively, and showed that the enzyme had a molecular weight of approximately 480,000. Several other groups also described the purification of a high molecular weight, ATP-dependent proteinase (Ismail and Gever; 1983; Dahlmann et al., 1983; Wilk and Orlowski, 1983; etc.).

Wilk and Orlowski (1983) were the first to show that this enzyme possessed different catalytic activities, and they named it the "multicatalytic proteinase complex". Dahlmann et al. (1985a) demonstrated that the enzyme possessed different proteolytic activities, and that the enzyme could be activated

by fatty acids or by SDS (Dahlmann et al., 1985b). They reported that the M_r of the enzyme was ~650,000, and that it dissociated into 8 subunits (M_r 25,000 to 32,000) when separated by SDS-polyacrylamide gel electrophoresis. Waxman et al. (1987) distinguished between the ATP-stimulated multicatalytic proteinase (~670 kDa) and an ATP-dependent ubiquitin-conjugate degrading enzyme (~1500 kDa) found in rabbit reticulocytes. They proposed that the two particles might be structurally related, and that the multicatalytic proteinase might be involved with the degradation of oxidatively damaged hemoglobin. Hough et al. (1987) also made a distinction between two high molecular weight proteinases from rabbit reticulocyte lysate. They also discussed the resemblance of the multicatalytic proteinase to the prosome.

Kopp et al. (1986) purified multicatalytic proteinase from rat skeletal muscle and reported that it had a cylindrical shape (11 x 16 nm) and that some of the particles appeared to be adsorbed to the grids in an upright position. They proposed that the molecule was composed of four rings of six subunits each, and that the round structures might be fragments of the molecule rather than upright particles.

Earlier reports of a complex 20-22S cylindrical particle include Shelton et al. (1970), Narayan and Rounds (1973), Smulson (1974), Domae et al. (1982), Kleinschmidt et al. (1983), and Harmon et al. (1983). Castaño et al. (1986) showed that a eukaryotic pre-tRNA 5′ processing endonuclease

copurified with a complex cylindrical particle. This preparation also consisted mainly of ring-shaped particles with some cylinders. The general consensus of these reports was that the particle appeared to be round with some rectangular profiles that were thought to be a stack of rings. However, Tanaka et al. (1988a) proposed that the rectangular particles were dimers of the enzyme produced during staining with uranyl acetate. Finally, Dahlmann et al. (1989) published the first pictures showing a relatively homogenous presentation of rectangular images.

Arrigo et al. (1988) showed that the multicatalytic proteinase was similar or identical to the prosome by several different criteria including: "size, polypeptide composition, immunological cross-reactivity, appearance in the electron microscope, radial symmetry of subunits, subcellular localization, and proteolytic activities". They did not, however, detect any RNA. They concluded that the two particles were identical, and, since the "prosome" appeared to play a role in intracellular protein breakdown, proposed that it be renamed the "proteasome". Falkenburg et al. (1988) also showed that the 19S-scRNP of Drosophila (Schuldt and Kloetzel, 1985) and the rat multicatalytic proteinase (Dahlmann et al., 1985a,b) were homologous, and concluded that all of the 19S cylindrical particles (eg. the multicatalytic proteinase, the prosome, miniparticles, aminoacyl synthetase, and eukaryotic pre-tRNA 5′ processing nuclease) were the same particle.

Since the change in nomenclature, and the general acceptance of the homology between the different 19S particles, there has been a tremendous increase in the study of the proteasome. It has been found in a wide variety of organisms and tissues (see reviews by Rivett, 1989a,b). It is generally considered to be ~700 kDa and to consist of 10-15 protein subunits ranging in size from ~21- ~35 kDa. The enzyme can cleave bonds on the carboxyl side of basic, hydrophobic, and acidic amino acid residues; and it appears to have three distinct types of catalytic activity: trypsin-like, chymotrypsin-like, and peptidyl-glutamyl peptide bond hydrolysis (Rivett, 1989c). There has been some debate as to whether it is ATP-dependent (Driscoll and Goldberg, 1989), or ATP-independent (Kuehn et al., 1989). The ATP dependence is apparently labile which may explain the discrepancy (Rivett, 1989a) and suggests that it may be ATP-dependent in vivo. It has been suggested that it might be a component of the ubiquitin-conjugate-degrading enzyme complex (Matthews et al., 1989), and this suggestion has been supported through studies by Driscoll and Goldberg (1990). The enzyme was also thought to have a latent form and an active form (McGuire et al., 1989; Mykles, 1989; McGuire and DeMartino, 1989).

Recently, investigators have changed from characterizing the protein composition and enzymatic activity of the proteasome to answering questions aimed at determining its biological significance. For example, recent studies have reported on

such diverse aspects as: the genes encoding the proteasome subunits; cell-specific accumulation of proteasomes during development; the apparent effect of the proteasome on translational activity in rabbit reticulocyte lysates; similarities in amino acid sequence among some of the proteasome subunits; the association of proteolytic activity with a particular subunit; and the co-purification of a small (70-90 nucleotide) RNA with the proteasome (Klein et al. (1990), Kuehn et al., (1990), Lee et al., (1990), Sato and Shiratsuchi, (1990), Skilton et al., (1991), (Haass et al., 1989,1990; Kumatori et al., 1990; Tanaka et al. 1990; Zwickl et al., 1991).

3.1.3 Hypothesis and objectives of this study

This work began with the hypothesis that ferritin might be the core protein component of the prosome. Ferritin is similar in size (~12 nm), mass (~450-500 kDa) and sedimentation value (19S) to the prosome, and it also appears to be morphologically similar by electron microscopy.

Preliminary results (Blaker et al., 1988) showed that ferritin was indeed a constituent of both the 20S and 19S fractions isolated from sucrose gradients, and initial attempts to distinguish between a ferritin-containing particle and a prosome particle were unsuccessful. Examination of the 19S fraction by electron microscopy revealed the presence of a particle that was similar to the prosome and yet was

apparently ferritin. The possibility that the ferritin in the 19S fraction was a contaminant from adjacent regions of the sucrose gradient was ruled out by repeated sucrose density gradient centrifugation of the 19S fraction. After 3 rounds of fractionation, the 19S fraction still contained about the same relative amounts of ferritin and a particle containing the prosome/proteasome protein subunits. I was finally able to separate ferritin from the particle containing the prosome/proteasome protein subunits by nondenaturing polyacrylamide gel electrophoresis, and the problem then became: 1) what was the significance of the presence of ferritin, and 2) why had ferritin not been recognized previously as a component of the prosome fraction?

At the same time that my studies were well underway, reports by Falkenburg et al. (1988) and Arrigo et al. (1988) were published that raised questions about the apparent homology between the prosome and the proteasome as well as the discrepancy in the apparent morphology of the two particles. I undertook to demonstrate conclusively that ferritin was a consistent constituent of the 19S "prosome" fraction and, to elucidate and resolve the controversy concerning the morphology of prosome and proteasome particles.

3.2 MATERIALS AND METHODS

3.2.1 Blood collection and radio-labelling of protein

Adult (~3 month-old) Japanese quail (Coturnix coturnix japonica) were made anemic by three successive daily subcutaneous injections of neutralized phenylhydrazine hydrochloride (15 mg/kg). Two days after the third injection, birds were killed by decapitation and blood was collected into 15 ml conical bottom tubes (Falcon 2095; Becton Dickinson, Lincoln Park, NJ) containing 0.1 ml Hepalean (Organon Canada, Toronto, ONT). The blood was centrifuged at 1,000 x g for 10 minutes, and the serum and leukocytes (visible as a whitish layer on top of the packed reticulocytes) were discarded. The packed reticulocytes were resuspended in 75 cm^2 Tissue Culture Flasks (Corning Glass Works, Corning, NY) containing methionine-free medium (1X Minimum Essential Medium, Eagle (Modified), with Earle's Salts and sodium bicarbonate, without methionine, without glutamine; Flow Laboratories, Irvine, Scotland), at a concentration of ~0.3-1.2 x 10^6 cells/ml, and incubated at 37°C for 30 minutes. The cells were radioactively labelled by the addition of 10 μCi/ml of L-^{35}S-methionine (Dupont-New England Nuclear, Boston, MA; specific activity >1000 Ci/mmole). Cells were labelled for 2 hours at 37°C with agitation of the flasks at 30 minute intervals.

3.2.2 Production of a postmitochondrial supernatant

Labelled cells were collected in 50 ml conical bottom tubes (Falcon 2098; Becton Dickinson) and pelleted at 1,000 x g for 10 minutes. The cells were washed with 5 volumes of methionine-free medium and repelleted at 1000 x g for 10 minutes. The labelled cells were lysed in ~10 volumes cold (4°C) lysing buffer (LB: 10 mM TEA-HCl (pH 7.4), 10 mM NaCl, 1 mM $MgCl_2$) and left on ice for 30 minutes. The lysate was centrifuged at 20,000 x g for 20 minutes, and the postmitochondrial supernatant was either used immediately or stored briefly at 4°C.

3.2.3 Production of a postribosomal pellet

Postmitochondrial supernatants were layered over a sucrose cushion (20% w/v in LB) and centrifuged at 340,000 x g (55.2Ti rotor (Beckman Instruments, Palo Alto, CA), 55000 rpm, 45 min, 4°C). The ribosomal pellets were discarded, and the postribosomal supernatants were collected and centrifuged at 340,000 x g (Beckman 55.2Ti rotor, 55000 rpm, 5 hours, 4°C). The resulting postribosomal pellets were resuspended in ~10 volumes LSB (10 mM TEA-HCl (pH 7.4), 50 mM NaCl, 1 mM $MgCl_2$), and stored at 4°C.

3.2.4 Production of a 20S fraction

Sucrose density gradients (10-25% w/w in LSB) were prepared using a BioComp Model 105 Gradient Maker (BioComp Instruments, Fredericton, NB). Aliquots of the resuspended postribosomal pellet (diluted with LSB to a protein concentration of ~1 μg/μl) were layered on the gradients and centrifuged at 141,000 x g (SW28 rotor (Beckman Instruments), 28000 rpm, 15 hours, 4°C). Tubes were fractionated using an ISCO Model 612 Density Gradient Fractionator (Instrumentation Specialties Co., Lincoln, NE), and the absorbance was monitored at 280 nm using an ISCO Model UA-4 Absorbance Monitor with a Type 4 detector (Instrumentation Specialty Co.). The sucrose was pumped out at 1.0 ml/min, and fractions of 1.2 ml were collected. Aliquots of each fraction were concentrated by centrifugation using Amicon Centricon 30 Microconcentrators (Amicon Corp., Danvers, MA) or Amicon CF25 Centriflo Ultrafiltration Membrane Cones (Amicon Corp.), and the concentrates were stored frozen for analysis by PAGE. The fractions of the 20S region (as determined by the separation of 16S and 23S rRNA (Boehringer Mannheim, Laval, Québec) on similarly prepared gradients) were pooled, diluted 2X with LSB and centrifuged at 340,000 x g (55.2Ti rotor (Beckman Instruments), 55000 rpm, 5 hours, 4°C). Pellets (referred to as the 20S fraction) were resuspended in LSB and stored at 4°C; in

some cases, aliquots were removed and stored at -20°C for analysis by PAGE.

3.2.5 Production of a 19S fraction

The resuspended 20S sample was made to contain 0.5 M NaCl (by the addition of a 2.0 M NaCl solution containing 10 mM TEA-HCl (pH 7.4), and 1 mM $MgCl_2$), layered over sucrose gradients (5-25% w/w in HSB: 10 mM TEA-HCl (pH 7.4), 500 mM NaCl, 1 mM $MgCl_2$), and centrifuged at 288,000 x g (SW41 rotor (Beckman Instruments), 41000 rpm, 10 hours, 4°C). Tubes were fractionated at a flow rate of 0.5 ml/min, and 0.4 ml fractions were collected. Absorbance was monitored at 280 nm. Aliquots of each fraction were concentrated by centrifugation using Amicon Centricon 30 Microconcentrators or Amicon CF25 Centriflo Ultrafiltration Membrane Cones (Amicon Corp.), and the concentrates were stored frozen for analysis by PAGE. The fractions of the 19S region (as determined by the separation of 16S and 23S rRNA (Boehringer Mannheim) on similarly prepared gradients) were pooled, diluted 2X with HSB and centrifuged at 340,000 x g (55.2Ti rotor (Beckman Instruments), 55000 rpm, 5 hours, 4°C). Pellets (referred to as the 19S fraction) were resuspended in LSB and stored at 4°C.

3.2.6 Proteinase-K treatment

In some cases, aliquots of samples were treated with proteinase-K by applying them to a proteinase-K CR-Column (BIO/CAN Scientific, Mississauga, ONT). The column was equilibrated with reaction buffer (50 mM Tris-HCl, pH 7.5; 5 mM NaCl). Samples (~50 µl) were prepared by adding one-third volume of 4X reaction buffer, and then applied to the column. The column was then incubated at room temperature for 5 minutes, after which it was eluted with several volumes of reaction buffer, and fractions were collected. Fractions containing undigested protein were pooled, concentrated using Amicon Centricon 30 Microconcentrators (Amicon Corp.), and stored at 4°C. The column was washed extensively with washing buffer (50 mM Tris-HCl, pH 7.5; 1.0 M NaCl), and then with storage buffer (50 mM Tris-HCl, pH 7.5) prior to being stored at 4°C.

3.2.7 Heat denaturation treatment

In some cases, aliquots of samples were placed in capped 1.5 ml Eppendorf centrifuge tubes and heat-denatured by placing them at 75°C for 15 minutes. The heat-treated samples were placed on ice for 30 minutes, centrifuged at ~5,000 x g for 5 minutes, and the supernatants (containing nondenatured protein) were transferred to separate tubes for storage (at

4°C) and analysis. If necessary, samples were concentrated using Amicon Centricon 30 Microconcentrators (Amicon Corp) prior to storage.

3.2.8 Measurement of radioactivity

3.2.8.1 Measurement by liquid scintillation counting

Radioactively-labelled protein in aliquots of samples was precipitated by the addition of 1/3 volume 50% trichloroacetic acid (w/v). Samples were mixed gently, placed on ice for 30 minutes, and centrifuged (5,000 x g, 10 minutes). The pellets were rinsed with diethylether, and recentrifuged. The ether was removed, and the pellets were dried *in vacuo* overnight. The dried pellets were resuspended in 80 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-MEt, and aliquots were removed for liquid scintillation counting.

Radioactivity was measured by a Beckman LS-255 liquid scintillation counter (Beckman Instruments, Palo Alto, CA) using a Triton X-100 - toluene - Omnifluor (Dupont-New England Nuclear, Dorval, Québec) cocktail.

3.2.8.2 Direct measurement

In the case of samples derived from the PMS (i.e. where there would not be any unincorporated ^{35}S-met), radioactivity

was measured directly by pipetting an aliquot of sample onto a ReadyCap (Beckman Instruments, Fullerton, CA). The radioactivity was measured by a Beckman LS-255 liquid scintillation counter and corrected for the efficiency of the counter for the ReadyCaps (~71%).

3.2.9 Polyacrylamide gel electrophoresis

3.2.9.1 Nondenaturing one-dimensional separations

All nondenaturing one-dimensional separations were done on Mini-Gels (83 x 55 x 1.5 mm; Bio-Rad Mini Protean II System; Bio-Rad Laboratories, Richmond, CA). All electrophoresis chemicals were Fio-Rad Electrophoresis Grade (Bio-Rad Laboratories). The native polyacrylamide gels consisted of a 5% separating slab gel (0.375M Tris-HCl, pH 8.9) overlaid with a 3% stacking gel (0.125M Tris-HCl, pH 6.8). Running buffer (25 mM Tris-HCl (pH 8.3), 192 mM glycine) was pre-cooled to ~10°C. Samples were made to contain 80 mM Tris-HCl (pH 6.8) by the addition of one-third volume of 4x native electrophoresis sample buffer (4X-NESB: 320 mM Tris-HCl (pH 6.8), 20% glycerol (w/v), 0.1% Bromphenol Blue). Samples were electrophoresed at 50V until the dye front had moved into the separating gel, and then at 125V until the dye front had just run off the bottom of the separating gel. Gels were stained in 0.1% (w/v) Coomassie Blue R (C.I. 42660; Sigma Chemical

Co., St. Louis, MO) in methanol:water:acetic acid (40:50:10), destained in methanol:water:acetic acid (10:80:10), and stored in water:acetic acid (90:10). The relative molecular mass (M_r) was estimated by comparing their distance of migration through the separating gel to a standard curve of Log(Molecular weight) (abscissa) vs Distance Migrated (ordinate) prepared using marker proteins of known molecular weight separated on the same gel (High Molecular Weight Calibration Kit: thyroglobulin dimer - 1,338,000 Da; thyroglobulin - 669,000 Da; ferritin - 440,000 Da; catalase - 232,000 Da; lactate dehydrogenase - 140,000 Da; bovine serum albumin - 67,000 Da; Pharmacia/LKB, Uppsala, Sweden).

3.2.9.2 Denaturing one-dimensional separations

Sodium dodecyl sulfate (SDS) denaturing one-dimensional polyacrylamide slab gels consisted of a 5-17.5% separating gel (0.375M Tris-HCl (pH 8.9), 0.1% (w/v) SDS) overlaid with a 3% stacking gel (0.125M Tris-HCl (pH 6.8), 0.1% (w/v) SDS). Running buffer contained 25 mM Tris-HCl (pH 8.3), 192 mM glycine, 0.1% (w/v) SDS. Samples were made to contain 80 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol (β-MEt), 10% (v/v) glycerol, 0.1% Bromphenol Blue, and applied to the gel. Samples were electrophoresed as follows: Mini-gels (83 x 55 x 1.5 mm separating gel) were run at 50V until the dye front had moved into the separating gel, and then at

150V until the dye front had run off; large gels (100 x 140 x 1.5 mm separating gel) were run at 5 mA/gel until the dye front had entered the separating gel, and then at 15 mA/gel until the dye front had run off. Gels were stained in 0.1% Coomassie Blue R as described previously. The relative molecular mass was estimated by comparing their distance of migration through the separating gel to a standard curve of Log(Molecular weight) (abscissa) vs Distance Migrated (ordinate) prepared using marker proteins of known molecular weight separated on the same gel (Low Molecular Weight Calibration Kit: phosphorylase b - 94,000 Da; bovine serum albumin - 67,000 Da; ovalbumin - 43,000 Da; carbonic anhydrase - 30,000 Da; soybean trypsin inhibitor - 20,000 Da; α-lactalbumin - 14,400 Da; Pharmacia/LKB).

In some cases, samples were separated by one-dimensional PAGE in the absence of a reducing agent but in the presence of detergent (either SDS or Sarkosyl). These gels consisted of a 5% separating gel (0.375M Tris-HCl (pH 8.9), 0.1% (w/v) detergent) overlaid with a 3% stacking gel (0.125M Tris-HCl (pH 6.8), 0.1% (w/v) detergent). The running buffer was 25 mM Tris-HCl (pH 8.3), 192 mM glycine, 0.1% (w/v) detergent. Samples were made up to 80 mM Tris-HCl (pH 6.8), 1% (w/v) detergent, 10% (v/v) glycerol, and applied to the gel. Electrophoresis was done at 75V until the dye front had run into the separating gel, and then at 125V until completed.

3.2.9.3 SDS-PAGE analysis of proteins separated on nondenaturing polyacrylamide gels

The 19S fraction was separated on one-dimensional nondenaturing gels as described. The position of the M_r ~877,000 and M_r ~562,000 components was determined by briefly staining lanes of the same gels that contained the high molecular weight markers and then excising the region of the 19S lane containing the component of interest. The excised gel pieces (~6 x 2 x 1.5 mm) were chopped into smaller pieces (~1.5 x 1 x 1.5 mm) with a razor blade and inserted into the well of a 5-17.5% SDS polyacrylamide gel. The well was filled with a solution of 80 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-MEt, 10% glycerol, and the pieces were allowed to equilibrate for 30 minutes prior to electrophoresis.

3.2.9.4 Two-dimensional separations

3.2.9.4.1 IEF-SDS-PAGE

Two-dimensional IEF-SDS-PAGE separation of proteins was done according to the method of O'Farrell (1975) with some minor modifications (see below). Separation was achieved by isoelectric focussing in the first dimension (i.e. proteins were separated on the basis of their pI) and by SDS-PAGE in the second dimension (i.e. separated on the basis of size).

The isoelectric focussing gel was a 3% polyacrylamide tube gel containing 9M urea, 2% Triton X-100, and 2% ampholines (Pharmacia/LKB, Bromma, Sweden; pH 3.5 to 10). These gels were pre-run (to establish the pH gradient) at 200V for 15 minutes, 300V for 30 minutes, and 400V for 30 minutes. The upper buffer (catholyte) was 0.25N NaOH, and the lower buffer (anolyte) was 0.2N H_3PO_4. The lower buffer was cooled by a water jacket to ~10°C. After the pre-run, the upper buffer was removed from the chamber and from the tops of the tube gels and the samples (made to contain 9M urea, 1% Triton X-100, 2% ampholines, and 5% β-MEt) were applied. Overlay buffer (4.5M urea, 1% ampholines) was applied, and subsequently covered with fresh upper buffer. Gels were electrophoresed at 400V for ~18 hrs, and then at 800V for 2 hrs. After electrophoresis, tube gels were extruded into a solution containing 80 mM Tris-HCl (pH 6.8), 2% SDS, and 5% β-MEt, and allowed to equilibrate for 30 minutes (with agitation). The tube gels were adhered to the top of slab gels (5-17.5% SDS-polyacrylamide separating gel with a 3% stacking gel) with 1% agarose (containing 80 mM Tris (pH 6.8), and 0.1% SDS) and run as previously described.

In some cases, samples were precipitated with trichloroacetic acid (TCA) prior to electrophoresis. Aliquots of sample were made to contain ~12% TCA by the addition of 1/3 volume of a 50% (w/v) solution of TCA. The sample was mixed gently, left on ice for 45 minutes, and pelleted in a micro-

fuge for 5 minutes (14,000 x g, 4°C). The pellets were washed with diethylether and spun briefly in the microfuge. The ether was removed by pipet, and the samples were dried in vacuo overnight. The dried pellets were resuspended in a solution of 9M urea, 1% NP-40 (or Triton X-100), 5% β-MEt, and 2% ampholines prior to separation by two-dimensional IEF/SDS-PAGE.

The isoelectric points of two-dimensionally-separated proteins were estimated from a standard curve prepared from a sample-free IEF gel that was run at the same time. The sample-free gel was extruded from the tube and cut into 5 mm segments. These segments were placed in 12 x 75 mm culture tubes containing 2.0 ml double-distilled water. The tubes were left to sit at room temperature for 1 hour, vortexed, and the pH of the solution was measured.

3.2.9.4.2 Native-SDS-PAGE

Samples were made to contain 80 mM Tris-HCl (pH 6.8), 10% (w/v) glycerol, 0.1% (w/v) bromphenol blue, and separated in the first dimension on a nondenaturing tube gel consisting of a 5% separating gel and a 3% stacking gel. Equilibration of the tube gel and the second dimension SDS-separation were done as described for two-dimensional IEF-SDS-PAGE.

3.2.10 Fluorography of polyacrylamide gels

Destained gels were soaked in three changes of dimethylsulfoxide (DMSO) to remove all traces of water and acetic acid. Each gel was then impregnated with 2,5-diphenyloxazole (PPO) by soaking in a 22% (w/v) solution of PPO in DMSO. The PPO-impregnated gels were immersed in a vast excess of water to precipitate the PPO, and dried down onto Whatman 3MM filter paper using a Bio-Rad Model 483 Gel Dryer (Bio-Rad Laboratories). The dried gels were taped to stiff cardboard and inserted into cassettes apposed to a piece of Kodak X-Omat RP X-ray film (preexposed to an absorbance (540 nm) of ~0.15 (Laskey and Mills, 1975)). Exposure of the film was carried out at -70°C.

3.2.11 Immunoblotting analysis of the 19S fraction

3.2.11.1 Transfer to nitrocellulose

Electrophoretically-separated proteins were transferred (using a Bio-Rad TransBlot Cell (Bio-Rad Laboratories)) at 60V for 8 hours to nitrocellulose (DuPont-New England Nuclear, Boston, MA) using 25 mM Tris (pH 8.3), 192 mM glycine, 20% v/v methanol as the transfer buffer (Towbin *et al.*, 1979). The nitrocellulose was blocked for 1 hour (at room temperature) in

a solution of 3% (w/v) Carnation Instant Skim Milk Powder (BLOTTO) in TBS (20 mM Tris-HCl (pH 7.5), 500 mM NaCl).

3.2.11.2 Immunoassay for ferritin

The blocked nitrocellulose was incubated in primary antibody solution containing rabbit anti-horse ferritin (Cappel Research Products; Organon Teknika, Scarborough, ONT) diluted 1:3,000 (in TBS) for 8 hours at room temperature, washed twice with TBS, and incubated in secondary antibody solution containing goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad Laboratories) diluted 1:1,000 (in TBS) for 2 hours at room temperature. Following the incubation with the secondary antibody the nitrocellulose was rinsed twice (5 minutes each) with TBS.

3.2.11.3 Immunoassay for prosome protein P27

Immunoassay for prosome protein P27 was done by the same method as the immunoassay for ferritin except that the primary antibody solution contained a monoclonal antibody to prosome protein P27 (Organon Teknika; Scarborough, ONT) diluted 1:1,000 (in TBS) and the secondary antibody solution contained goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad Laboratories) diluted 1:1,000 (in TBS).

3.2.11.4 Colour development

Cross-reaction of primary and secondary antibodies in both systems was visualized using 4-chloro-1-naphthol (4-CN; Bio-Rad Laboratories) as a substrate for the horseradish peroxidase conjugated to the secondary antibody. A solution of 60 mg of 4-CN in 20 ml of methanol was added to a solution of 100 ml TBS containing 60 µl 30% H_2O_2, immediately prior to use. The rinsed membranes were incubated in the resulting solution for ~30 minutes (in the dark), and colour development was stopped by rinsing in distilled water. The developed immunoblots were stored in water (in the dark) until photographed.

3.2.12 Protease assay

3.2.12.1 Fluorimetric assay

The 20S fraction, the 19S fraction, and the 19S proteinase-K-treated fraction were assayed for protease activity by a modification of the method of Hough *et al*. (1987). The concentrations of the three samples were adjusted so that 50 µl represented 1.0 % of the total volume of the sample.

Samples (5 and 20 µl aliquots) were placed in separate wells of 96-well tissue culture plates (Costar, Cambridge, MA), and an equal volume of 2X protease assay buffer (PAB; 1X

PAB: 50 mM Tris-HCl (pH 7.8), 25 mM KCl, 10 mM NaCl, 1.1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 10% glycerol (v/v)) was added to each well. Samples were made up to 100 µl with 1X PAB (control wells contained 100 µl PAB), and the covered plate was incubated for 10 minutes at 37°C. Substrate (1.0 µl of a 0.1 mM solution of N-succinyl-leu-leu-val-tyr-7-amido-4-methyl-coumarin (Sigma Chemical Co.) in 1X PAB) was added, and the solution was mixed. Incubation was continued for 30 minutes at 37°C. The plate was immediately irradiated on a Spectroline Transilluminator (Model TR-302; Spectroline Corp., Westbury, NY) at 302 nm, and photographed using an MP-4 Land Camera (Polaroid, Cambridge, MA) with 665 Positive/Negative Professional Film (Polaroid).

3.2.12.2 Radioactive assay

The M_r ~877,000 and M_r ~562,000 components were electroeluted from nondenaturing polyacrylamide gels and their purity and integrity were checked by separating an aliquot on a nondenaturing gel.

Samples of the purified components (5 µg) were made up to 450 µl by the addition of stock solutions of Tris-HCl (pH 7.5), NaCl, $MgCl_2$, and DTT so that the final concentration of a 500 µl solution would be 50 mM Tris-HCl (pH 7.5), 20 mM NaCl, 2.5 mM $MgCl_2$, 2.5 mM DTT. The assay was initiated by the addition of 50 µg of ^{3}H-α-methylcasein (1 µg/µl) and

incubated at 37°C for 2.5 hours. In some cases, samples were incubated in the presence of either ATP (5 mM) or poly-L-lysine (1 μg/μl). The reaction was stopped by the addition of 40 μl BSA (30 mg/ml) and 60 μl TCA (100% w/v), and chilled on ice for 1 hour. Samples were transferred to 1.5 ml Eppendorf tubes, centrifuged at 12,000 rpm for 5 minutes in an Eppendorf Microfuge, and 400 μl samples were removed and radioactivity was measured by liquid scintillation counting. Proteolytic activity was measured (as disintegrations per minute; dpm) as the degradation of α-methylcasein to products soluble in 10% (w/v) TCA. One unit of enzyme activity is the amount of enzyme which solubilizes 1 μg casein per hour and is expressed units/μg protein.

3.2.13 Electron microscopy

3.2.13.1 Preparation of negative stain solution

A 5 ml solution of 3% (w/v) phosphotungstic acid (PTA) was prepared, and to it was added 10 μl of 1% (w/v) Phenol Red. The pH of the solution was neutralized by the addition of 0.1N NaOH until the solution was orange. The PTA solution was then sterile-filtered and stored at room temperature. Fresh stain was prepared as required.

3.2.13.2 Negative staining of samples

Samples were stained as whole mounts by either a one-step or a two-step protocol (see below). Samples were applied to either carbon-shadowed, Formvar-coated copper grids, or to carbon-film-coated copper grids.

3.2.13.2.1 One-step staining protocol

An aliquot of sample was mixed with an equal volume of PTA solution, applied to a grid, and immediately blotted away using a small piece of Whatman #1 filter paper.

3.2.13.2.2 Two-step staining protocol

An aliquot of sample was applied directly onto a grid, and immediately blotted away using a small piece of Whatman #1 filter paper. The grid was air dried for ~30 seconds. The grid was then stained by the application of an aliquot of PTA solution which was immediately blotted away with filter paper.

3.2.13.3 Electron microscopic examination

Grids were examined at 60 kV using a Philips 201 EM, and photographed with Fine Grain Release Positive film (35 mm; Eastman Kodak, Rochester, NY); or examined at 80 kV with a

Philips 300 EM, and photographed with SO163 Electron Image Film (4" x 3.25"; Eastman Kodak, Rochester, NY).

3.2.14 Image enhancement

Electron micrographic images on SO-163 Electron Image film (Eastman Kodak) were digitized directly by the image processing software of a Bio-Rad MRC-600 Confocal Imaging System (Bio-Rad Microscience, Hertfordshire, England) using a Panasonic WV-BD-400 Video Camera (Matsushita Communication Industrial Co., Japan). For the rectangular profile, ten random images were recorded and each image was rotated 180° for a total of 20 images. For the end-on profile, twenty random images were recorded. The 20 images of each set were aligned using the video display of the computer and the image processing software was used to generate a composite image. The final image was reversed from negative to positive by the software and printed by a SONY UP-5000 video printer (Sony Corporation, Japan).

3.2.15 RNase treatment

RNase A (Boehringer Mannheim) was added to aliquots of the 19S and proteinase-K-treated 19S fractions (final concentration 1.0 μg/μl) and incubated at 37°C for 30 minutes.

Samples made to contain 80 mM Tris-HCl and were separated on a nondenaturing polyacrylamide gel.

3.2.16 Analysis of RNA of the 35S fraction

3.2.16.1 Phenol extraction of RNA

An aliquot of the 35S fraction was mixed in a 1.5 ml Eppendorf tube with an equal volume of phenol/chloroform/isoamyl alcohol (24:24:1) and centrifuged at 14,000 x g for 1 minute (at 4°C). The aqueous phase was collected and an equal volume of chloroform/isoamyl alcohol (24:1) was added. The sample was mixed and centrifuged at 14,000 x g for 1 minute (4°C). The aqueous phase was collected and made up to 0.3 M sodium acetate (pH 6.0) and 2.5 volumes of cold 95% ethanol was added. The sample was mixed gently and placed at -70°C for 2 hours. The precipitated RNA was pelleted at 14,000 x g for 10 minutes (4°C). The pellet was rinsed with 80% ethanol and centrifuged at 14,000 x g for 2 minutes. The supernatant was discarded and the pellet was dried *in vacuo*. The dried pellet was resuspended in a minimum volume of sterile, glass-distilled water.

3.2.16.2 Electrophoretic separation of RNA

3.2.16.2.1 Urea/acrylamide gel electrophoresis

Aliquots (~5.0 µl) of sample were mixed with ~20 µl of loading buffer (95% (v/v) deionized formamide, 5% (v/v) 20X TBE (1X TBE= 89 mM Tris-borate (pH 8.3), 2.5 mM EDTA), 0.01% (w/v) bromphenol blue, 0.01% (w/v) xylene cyanol), heated at 50°C for 10 minutes, and separated on an 8% polyacrylamide Mini-gel containing 7 M urea and 1X TBE (running buffer was 1X TBE). The gel was electrophoresed at 100V until the bromphenol blue was ~1 cm from the bottom. The gel was stained in 1X TBE containing ethidium bromide (1 µg/ml) for 30 minutes and destained (briefly) in 1X TBE. The destained gel was irradiated using a 302 nm Transilluminator (Spectroline Corp.) and photographed with an MP-4 Land Camera (Polaroid) using 665 Positive/Negative Professional Film (Polaroid). Lanes adjacent to the sample contained 4S yeast tRNA (~80 nucleotides; Boehringer Mannheim) for use as a size marker.

3.2.16.2.2 Agarose gel electrophoresis

Aliquots (6.25 µl) of sample were mixed with 1.0 µl 10X MOPS (N-morpholino-propanesulfonic acid; 1X MOPS = 20 mM (pH 7.0)), 2.75 µl formaldehyde (37% w/v) and 1.0 µl loading buffer (50% (v/v) glycerol, 1 mM EDTA (pH 8.0), 0.25% (w/v)

bromphenol blue, 0.25% (w/v) xylene cyanol, heated at 65°C for 5 minutes and cooled on ice. Samples were separated on 1% agarose gels containing 7.7% formaldehyde, using 1X MOPS as running buffer. One lane of the gel contained a sample of an RNA ladder (BRL). This lane was stained with ethidium bromide for use in determining the size (i.e. number of nucleotides) of bands of interest.

3.2.17 RNA Hybridization

RNA electrophoretically-separated on agarose gels was vacuum-transferred (2016 Vacugene Vacuum Blotting Unit; Pharmacia/LKB) to ZetaProbe membrane (Bio-Rad). The membrane was then placed in a PR-800 HybridEase chamber (Canberra-Packard Canada Ltd., London, ONT) and prehybridization solution (50% (v/v) formamide, 1.5X SSPE (20X SSPE = 3.0 M NaCl, 0.2 M Na_2HPO_4 (pH 7.0), 0.02 M EDTA), 1% (w/v) SDS, 0.5% (w/v) BLOTTO, 0.5 mg/ml herring sperm DNA (Sigma)) was added. Prehybridization was done at 50°C for 4 hours.

The prehybridization solution was replaced with hybridization solution (prehybridization solution plus 10% (w/v) dextran sulfate (Pharmacia)) containing a quail β-globin cDNA probe. This cDNA contains the complete coding region (613 base pairs) for quail β-globin plus 80 nucleotides of the 5'-untranslated region and 100 nucleotides of the 3'-untranslated region (Dr. B.G. Atkinson, unpublished). Using a random

primer labelling kit (BRL) the probe was labelled to a specific activity >1 X 10^9 cpm/μg with α-^{32}P-dCTP (DuPont; specific activity >3000 μCi/mmole). Hybridization was done at 50°C for 20 hours.

The membrane was rinsed with 2X SSC (20X SSC = 3 M NaCl, 0.3 M sodium citrate) followed by 15 minute washes (once each) of 2X SSC (containing 0.1% (w/v) SDS), 0.5X SSC (containing 0.1% (w.v) SDS, and 0.1X SSC (containing 0.1% (w.v) SDS), all at room temperature. The final stringency wash was done in 0.1X SSC (containing 1.0% (w/v) SDS) at 60°C for 30 minutes. The membrane was dried in vacuo at 80°C, wrapped with Saran-Wrap, and placed in apposition to a piece of Kodak XR-OMAT film preflashed to an absorbance (540 nm) of 0.15 (Laskey and Mills, 1975) and backed by an intensifier screen (DuPont). Exposure of the film was carried out at -70°C.

3.3 RESULTS

3.3.1 Origin of the 20S fraction

Reticulocytes from anemic quail were isolated and lysed as described (see Materials and Methods). A 20,000 x g post-mitochondrial supernatant was obtained from the reticulocyte lysate, layered over a sucrose pad and centrifuged to remove the ribosomes (see Appendix 1 for an experimental flowchart). The postribosomal supernatant was centrifuged at 340,000 x g, and the resulting postribosomal pellet was resuspended in LSB. This sample was diluted to a protein concentration of ~1 μg/μl and layered onto 10-25% (w/w in LSB) sucrose gradients. Fractions were collected and analyzed by one-dimensional SDS-PAGE (Figure 3.1). The fractions of the 20S region (Fractions 12 to 15; Figure 3.1, Lanes 6 to 8; determined by the use of 16S and 23S rRNA markers separated on similarly prepared gradients) were pooled and centrifuged at 340,000 x g. The pellet was resuspended in LSB and is referred to as the 20S fraction.

3.3.2 Analysis of the 20S fraction

The 20S fraction was treated with 0.5M NaCl and separated by sucrose density gradient ultracentrifugation (Figure 3.2, Panel A). One-dimensional SDS-PAGE analysis of the gradient

Figure 3.1. Separation of the 20S fraction from the resuspended postribosomal pellet (PRP) isolated from quail reticulocyte lysate. Equal volumes of gradient fractions were separated by one-dimensional SDS-PAGE (N.B. the samples of lanes 6 to 15 were concentrated 8X relative to lanes 1 to 5). Samples were not boiled in the presence of SDS and β-mercaptoethanol prior to electrophoresis. Panel A, Coomassie-blue stain . Panel B, fluorogram of Panel A. The proteins of the PRP are distributed over much of the gradient (Lane 1, top of the gradient; Lane 15, bottom of the gradient). The 20S region (Lanes 6-8; as determined by separation of 16S and 23S rRNA on similarly prepared gradients) was pooled, concentrated by ultracentrifugation, and resuspended to produce the 20S fraction. Marker proteins of relative molecular mass ($M_r \times 10^{-3}$) are indicated. See Materials and Methods for details.

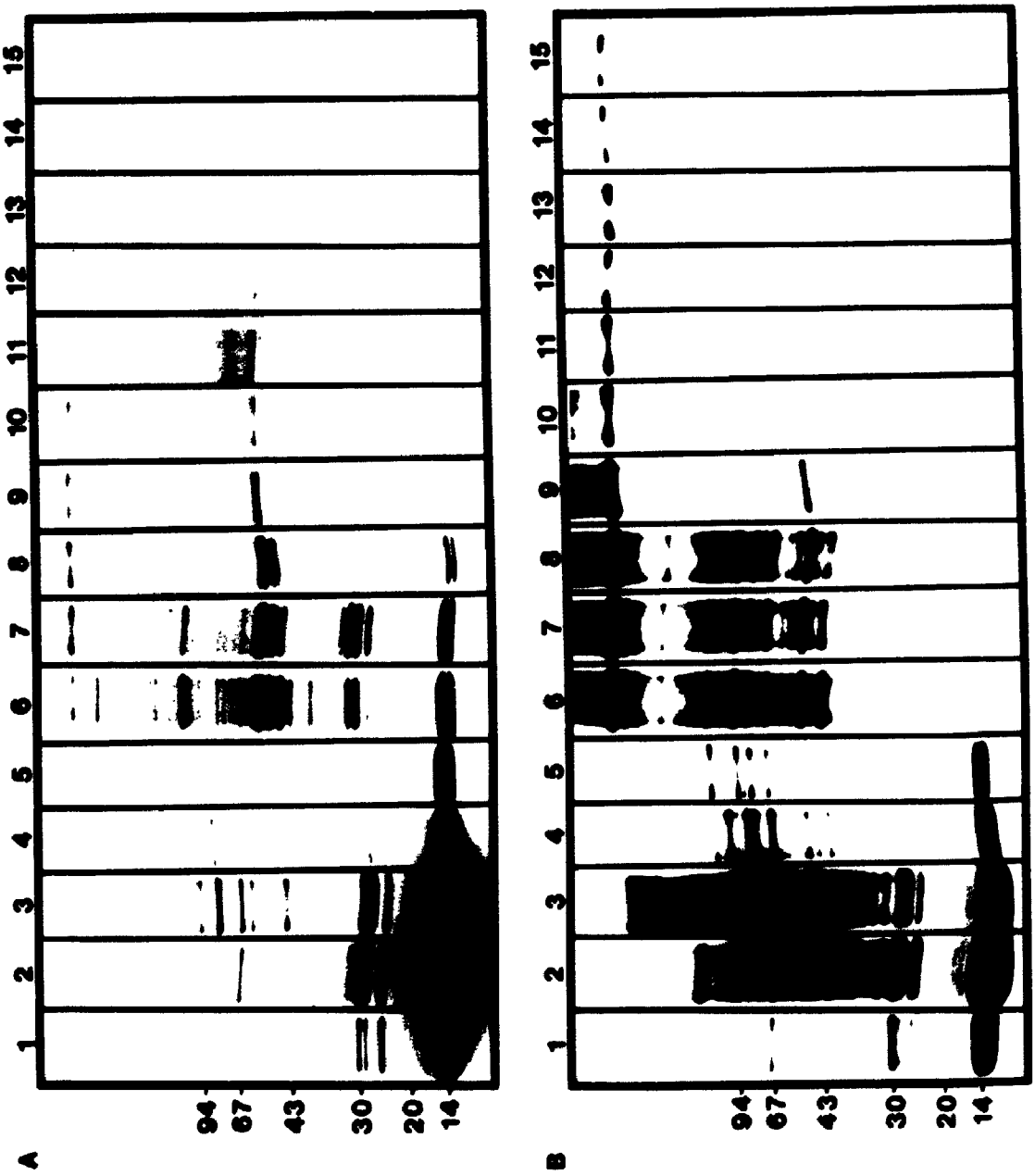

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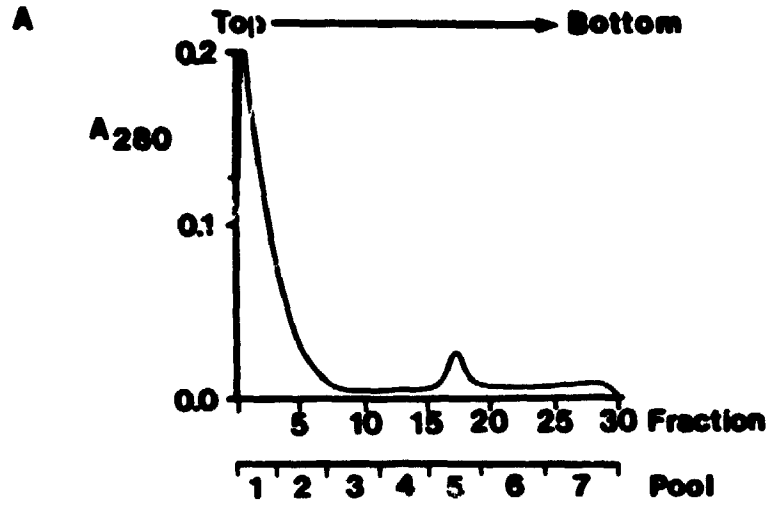
Figure 3.2. Separation of the 19S region from the high-salt-treated 20S fraction isolated from quail reticulocyte postribosomal supernatant. Panel A. Typical absorbance profile of a high-salt-treated 20S fraction separated by sucrose density gradient ultracentrifugation. Panels B and C. Equal volumes of pooled gradient fractions were separated by one-dimensional SDS-PAGE (samples were not boiled in the presence of SDS and β-mercaptoethanol prior to electrophoresis). Panel B, Coomassie-blue stain. Panel C, fluorogram of Panel B. The proteins of the 20S fraction are distributed over much of the gradient (Lane 1, top of the gradient; Lane 7, bottom of the gradient). Lane 1, pool 1 (fractions 1 to 3); lane 2, pool 2 (fractions 4 to 7); lane 3, pool 3 (fractions 8 to 11); lane 4, pool 4 (fractions 12 to 15); lane 5, pool 5 (fractions 16-19); lane 6, pool 6 (fractions 20 to 24); lane 7, pool 7 (fractions 25 to 29). The 19S region (Lane 6) contains a well-labelled >450 kDa protein and a set of smaller proteins (~22--32 kDa), as well as some intermediate-sized proteins (eg. at ~48, ~92, and ~120 kDa; see arrowheads). Marker proteins of relative molecular mass ($M_r \times 10^{-3}$) are indicated. See Materials and Methods for details.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Bottom
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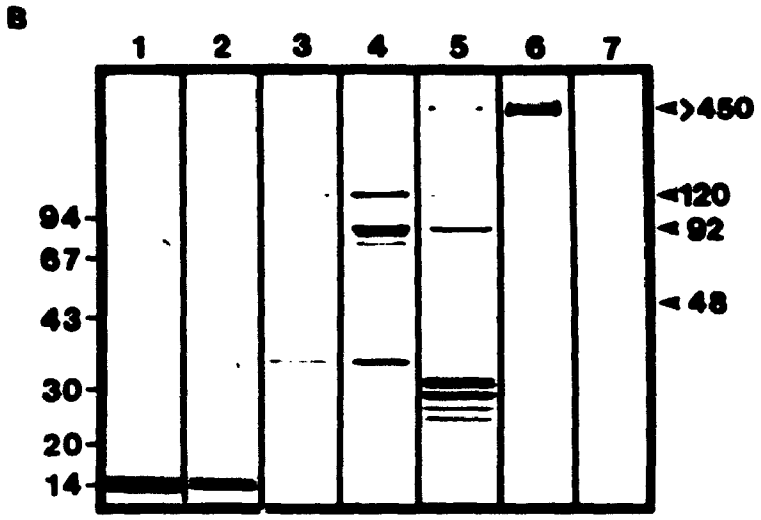
0.0
5 10 15 20 25 30 Fraction
1 2 3 4 5 6 7 Pool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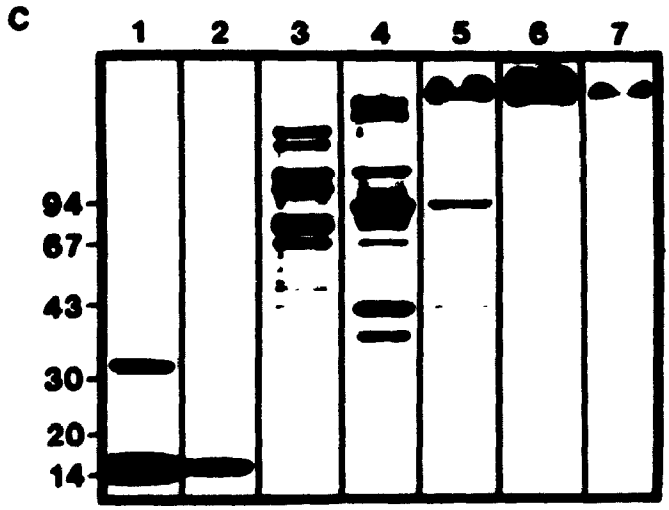
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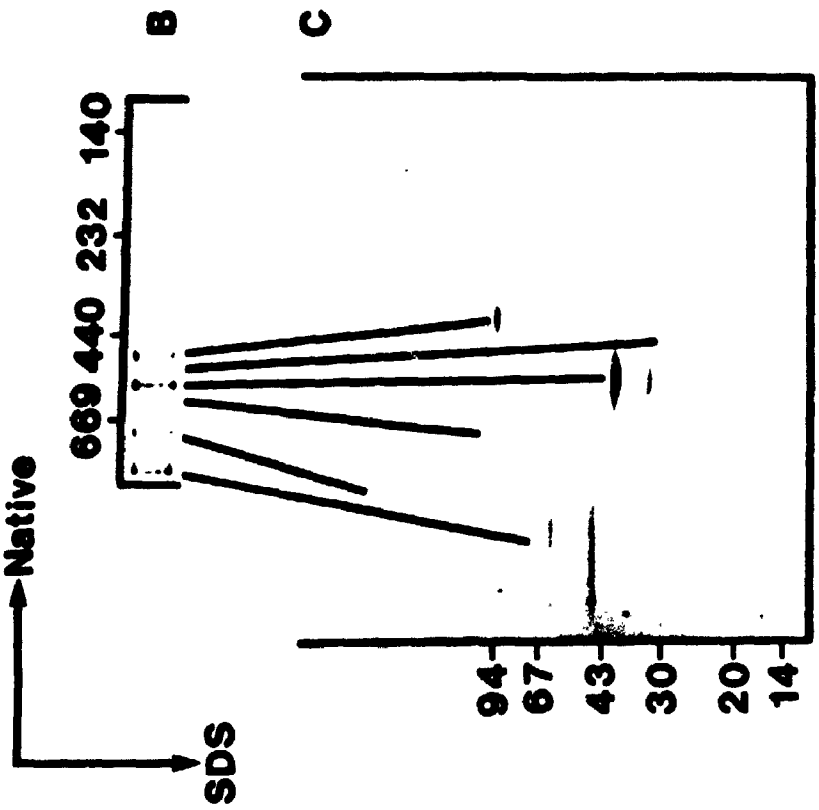
fractions reveals that the proteins of the 20S fraction are distributed throughout the gradient (Figure 3.2, Panels B and C). The peak at approximately 19S (Figure 3.2, Panel A, fractions 16 to 19) contains a ~22 kDa to ~32 kDa set of proteins (Figure 3.2, Panel B, Lane 5) that do not appear to contain any radioactive label (Figure 3.2, Panel C, Lane 5). There are other proteins present in the 19S region which overlap from adjacent regions of the gradient. These include components of ~48, ~92, ~120, and >450 kDa (see arrowheads, Figure 3.2, Panel B). The >450 kDa protein is very well labelled and is detected in all fractions taken from the lower half of the gradient.

3.3.3 Nondenaturing PAGE analysis of the 20S fraction

One-dimensional nondenaturing PAGE shows that the 20S fraction contains several high molecular weight protein-containing particles ranging in relative mobilities from M_r ~510,000 to M_r ~1,400,000 (Figure 3.3, Panel A). Two-dimensional nondenaturing/SDS-PAGE shows that each of these particles is composed of distinct protein subunits (Figure 3.3, Panel C).

Figure 3.3. Electrophoretic analysis of the 20S fraction. Panels A and B, one-dimensional analysis. An aliquot of the 20S fraction was separated on a 5% nondenaturing slab gel. Panel C, two-dimensional analysis. An aliquot of the 20S fraction was separated in the first dimension on a 5% nondenaturing tube gel, and in the second dimension on a 5-17.5% SDS gel. The bands of the gel pictured in panels A and B correspond to the spots in panel C. The protein composition of each band of panels A and B was determined by the electroelution of corresponding regions from an identical unstained lane and subsequent separation of the concentrated proteins on a one-dimensional 5-17.5% SDS polyacrylamide gel (not shown). The 20S fraction contains as many as six distinct protein-containing components of relative mobilities (M_r) of approximately 1 400 000, 877 000, 630 000, 600 000, 562 000, and 510 000. Marker proteins of relative molecular mass ($M_r \times 10^{-3}$) are indicated.


B
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140
232
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SDS
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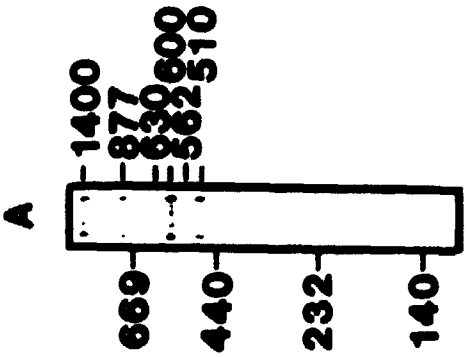
20
14


A
1400
877
630
582
510
669
440
232
140

3.3.4 One-dimensional SDS-PAGE comparison of the 20S fraction, the 19S region, and the 19S fraction

The fractions of the SW41 gradient that contained the 19S region (fractions 16-19; see Figure 3.2, Panel B) were pooled and concentrated by ultracentrifugation. The pellet was resuspended in LSB, and is referred to as the 19S fraction. One-dimensional SDS-PAGE analysis shows that the pelleted 19S fraction (Figure 3.4, lanes 5 and 6) contains only the ~22--32 kDa set of proteins and the >450 kDa protein of the 19S region (Figure 3.4, lanes 3 and 4); the ~48, ~92, and ~120 kDa proteins detected in the 20S fraction (Figure 3.4, lanes 1 and 2) and in aliquots from the 19S region of the SW41 gradient are not detectable in the pelleted 19S fraction. Therefore, the 19S fraction of quail RBCs contains particle(s) which pellet at 340,000 x g (for 5 hours) and are made up of a set of poorly labelled, low molecular weight protein components and a well-labelled, >450 kDa protein component.

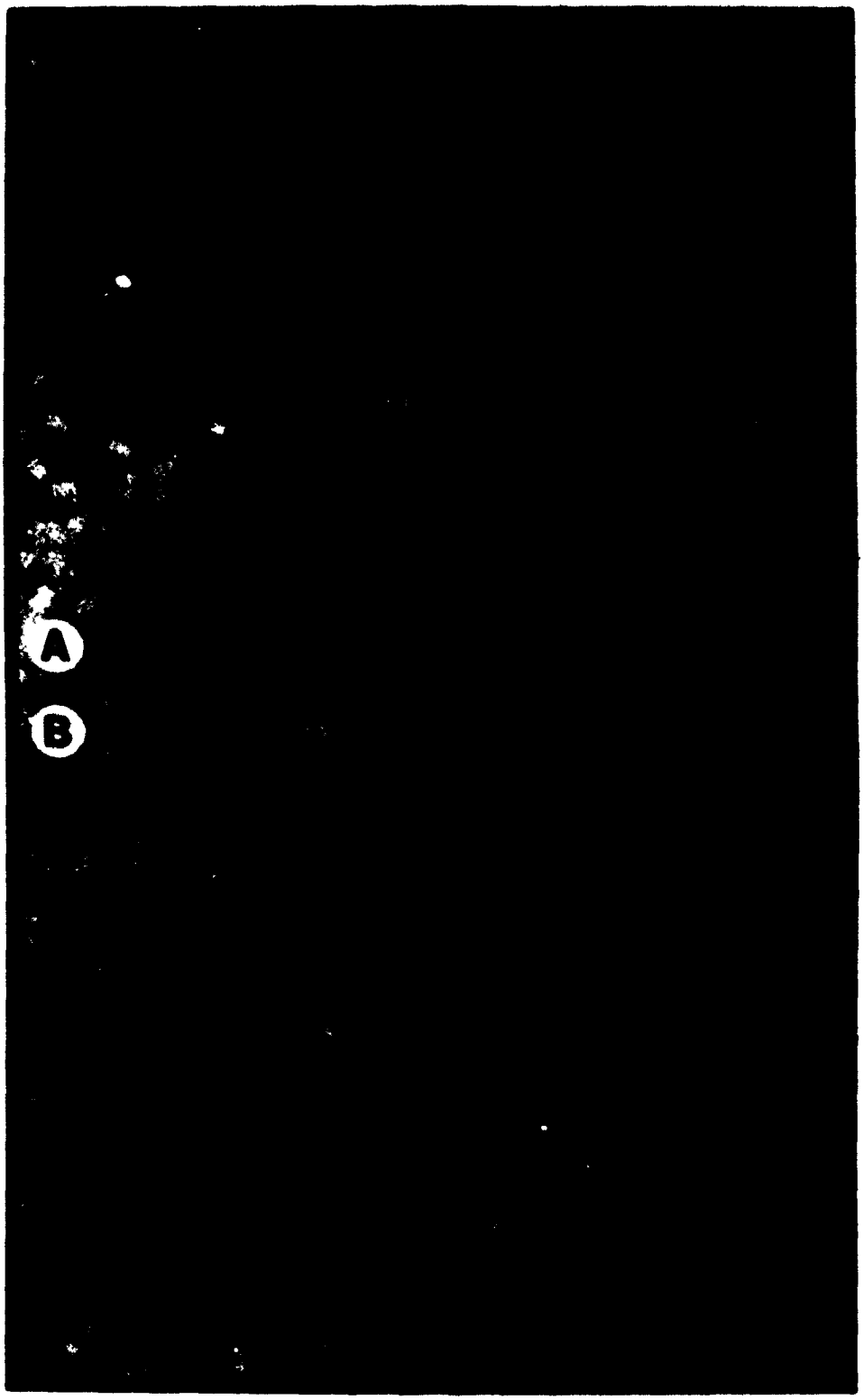
3.3.5 Electron microscopic analysis of the 19S fraction

Electron microscopic analysis of the 19S fraction negatively stained by a one-step stain protocol (see Materials and Methods) reveals that it contains three morphologically distinct particles (Figure 3.5). Most of the particles (90-95%) are rectangular in profile (~10 X 15 nm); the remainder

Figure 3.4. One-dimensional SDS-PAGE comparison of the 20S fraction, the 19S region, and the 19S fraction. Lanes 1 and 2 contain samples of the pelleted 20S fraction, lanes 3 and 4 contain samples taken directly from the 19S region of the SW41 sucrose gradient, and lanes 5 and 6 contain samples of the pelleted 19S fraction. Lanes 1, 3, and 5 are Coomassie-blue-stained and lanes 2, 4, and 6 are fluorograms of the gels shown in lanes 1, 3, and 5 respectively. Samples were made to contain 2% SDS and 5% β-mercaptoethanol and applied directly to gels. The pelleted 19S fraction is free of the ~48, ~92, and ~120 kDa protein components of the 19S region (see arrows), but the ~22-~32 kDa set of proteins and the >450 kDa protein remain. Marker proteins of relative molecular mass ($M_r \times 10^{-3}$) are indicated.


1
2
3
4
5
6
94-
67-
43-
30-
20-
14-
450
120
92
48

Figure 3.5. Electron microscopic analysis of the 19S fraction isolated from quail reticulocyte PRS. Panel A. Low magnification view of a negatively stained whole mount (X 200,000). Panel B. High magnification view of a negatively stained whole mount (X 700,000). The 19S fraction contains three distinct profiles. The major component is a particle with a rectangular profile (~10 X ~15 nm) that constitutes 90-95% of the sample (1). There are also two minor components (each constituting ~3-4% of the sample): a round particle that is 12-13 nm in diameter (2), and a slightly smaller round particle that is ~10.5 nm in diameter (3). Both preparations were negatively stained by a one-step protocol. See Materials and Methods for details. Scale bar = 20 nm.


A
B

consist of approximately equal numbers (~3-4% each) of two round particles: one is 12-13 nm in diameter, the other is ~10.5 nm in diameter. Both of the round particles may be uniformly bright, or may have a dark center of varying size.

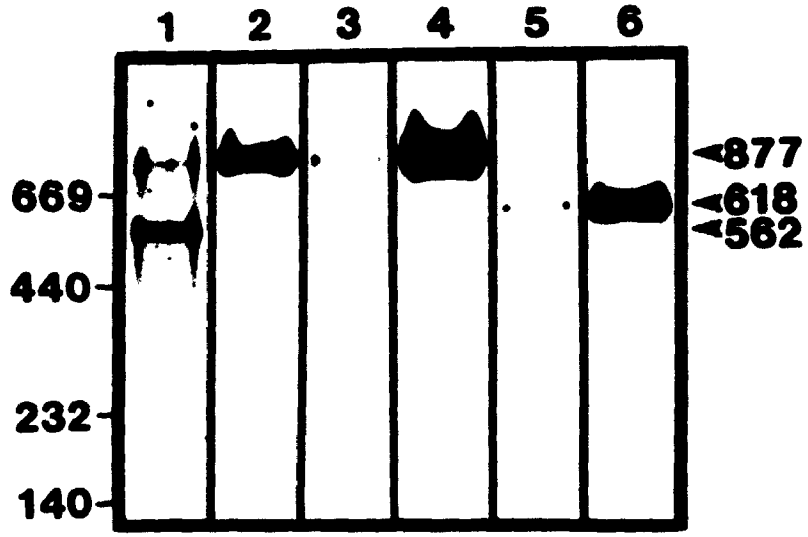
3.3.6 One-dimensional nondenaturing PAGE analysis of the 19S fraction

Analysis of the 19S fraction by one-dimensional nondenaturing PAGE shows that there are only two detectable high molecular weight components (Figure 3.6, Lane 1). The major component migrates at a relative molecular mass (M_r) of ~562,000, and the minor component migrates at M_r ~877,000. The M_r ~877,000 component is well-labelled with ^{35}S-methionine, while the M_r ~562,000 component appears to contain little or no label (Figure 3.6, Lane 2).

One-dimensional nondenaturing PAGE analysis of the proteinase-K-treated 19S fraction shows that this treatment eliminates the M_r 562,000 component, and only the M_r 877,000 component is recovered (Figure 3.6, lanes 3 and 4).

Analysis of the heat-denatured 19S fraction by one-dimensional nondenaturing PAGE reveals that the M_r ~562,000 component has been denatured and that the well-labelled particle remains in the supernatant (Figure 3.6, lanes 5 and 6). However, the mobility of this component has changed to M_r ~618,000.

Figure 3.6. One-dimensional nondenaturing PAGE analysis of the 19S fraction. Lanes 1 and 2, 19S fraction; lanes 3 and 4, proteinase-K-treated 19S fraction; lanes 5 and 6, heat denatured 19S fraction. Lanes 1, 3, and 5, Coomassie-blue stain; lanes 2, 4, and 6, fluorograms of lanes 1, 3, and 5, respectively. All samples were separated in the absence of detergent and reducing agent. The 19S fraction contains only two detectable high molecular weight components: (1) an apparently unlabelled component that migrates at a relative molecular mass (M_r) of ~562,000; and (2) a well-labelled component that migrates at a relative molecular mass of ~877,000. Proteinase-K treatment of the 19S fraction eliminates the M_r ~562,000 component from the soluble phase, and only the labelled M_r ~877,000 component remains. Heat denaturation also eliminates the M_r ~562,000 component from the soluble phase, and causes a shift in the mobility of the well-labelled component from M_r ~877,000 to M_r ~618,000. Marker proteins of relative molecular mass ($M_r \times 10^{-3}$) are indicated.


1
2
3
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5
6
669
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877
618
562

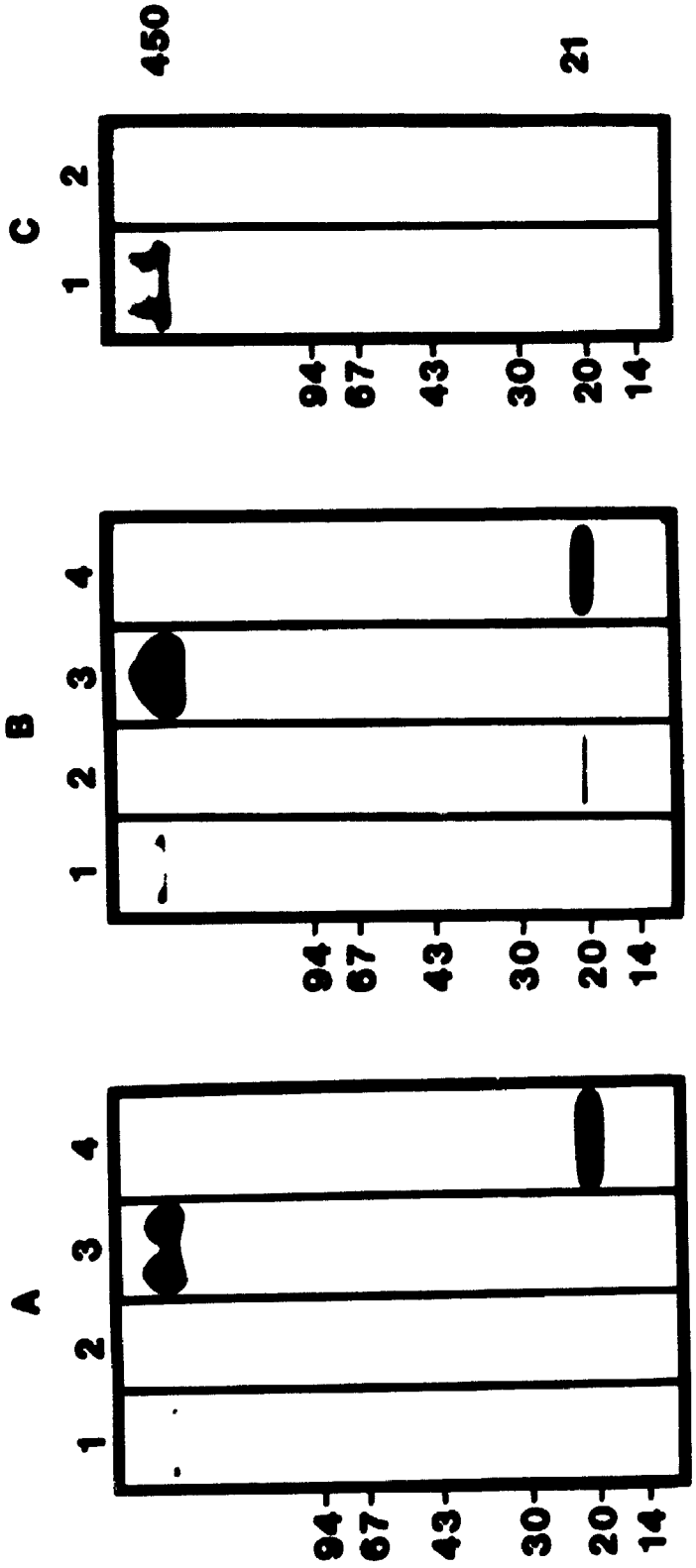
3.3.7 One-dimensional SDS-PAGE analysis of the 19S fraction after proteinase-K-treatment or heat denaturation

Analysis of the proteinase-K-treated 19S fraction by one-dimensional SDS-PAGE reveals that the M_r ~877,000 proteinase-K-resistant particle contains the >450 kDa protein (Figure 3.7, Panel A, lanes 1 and 3) and that the ~22-32 kDa set of proteins has been eliminated. The >450 kDa protein of the M_r 877,000 proteinase-K-resistant particle separates into 21 kDa subunits when boiled in the presence of SDS and β-mercaptoethanol (β-MEt) prior to SDS-PAGE (Figure 3.7, Panel A, lanes 2 and 4). When the 19S sample is heated at 75-80° C for 10 minutes, the well-labelled particle that remains in supernatant is still comprised of a >450 kDa protein that separates into 21 kDa subunits when boiled in the presence of SDS and β-mercaptoethanol prior to electrophoresis (Figure 3.7, Panel B), even though its mobility on nondenaturing gels has changed (see Figure 3.6, lanes 5 and 6).

The >450 kDa protein does not contain any detectable iron (not shown).

The proteinase-K-treated 19S fraction was separated by one-dimensional SDS-PAGE, and the electrophoretically separated proteins were transferred to nitrocellulose for immunoassay. Both the >450 kDa protein and its 21 kDa subunits cross-react with antibodies to horse ferritin (Figure 3.7, Panel C).

Figure 3.7. One-dimensional SDS-PAGE analysis of the proteinase-K-treated 19S fraction and the heat-denatured 19S fraction. Panels A and B, lanes 1 and 2, Coomassie-blue stain; lanes 3 and 4, fluorograms of lanes 1 and 2, respectively. Lanes 1 and 3, samples were not boiled in the presence of SDS and β-mercaptoethanol prior to electrophoresis; lanes 2 and 4, samples were boiled in the presence of SDS and β-mercaptoethanol prior to electrophoresis. Panel A, proteinase-K-treated 19S fraction. The proteinase-K-resistant M_r ~877,000 component contains the >450 kDa protein of the 19S fraction, and this protein separates into 21 kDa subunits when boiled prior to electrophoresis. Panel B, heat-denatured 19S fraction. The M_r ~618,000 component that remains in the supernatant still contains the >450 kDa protein of the 19S fraction, and this protein separates into 21 kDa subunits when boiled prior to electrophoresis. Panel C, immunoblot analysis of the >450 kDa protein. Aliquots of the proteinase-K-treated 19S fraction were separated by one-dimensional SDS-PAGE, electrophoretically transferred to nitrocellulose, and incubated with antibody to horse ferritin. Lane 1, sample not boiled prior to electrophoresis. Lane 2, sample boiled prior to electrophoresis. Both the >450 kDa protein and its 21 kDa subunit crossreact with antibody to horse ferritin. Marker proteins of relative molecular mass ($M_r \times 10^{-3}$) are indicated.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20
14
450
21

3.3.8 Electron microscopic analysis of the proteinase-K-treated 19S fraction

Electron microscopic examination of the proteinase-K-treated 19S fraction reveals that only the 12-13 nm particle remains (Figure 3.8, Panel A); both the particle with the rectangular profile and the 10.5 nm particle have been destroyed by the proteinase-K treatment. The 12-13 nm particle is also indistinguishable from proteinase-K-treated horse ferritin (Figure 3.8, Panel B) and quail red blood cell ferritin isolated by conventional means (Atkinson *et al.*, 1989).

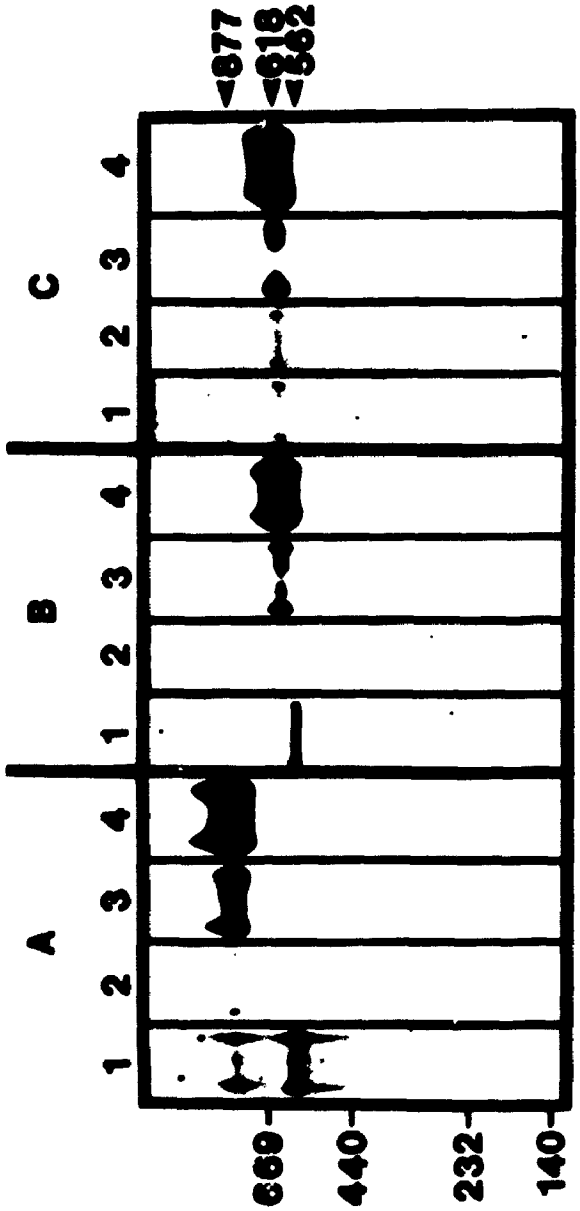
3.3.9 One-dimensional PAGE analysis of the 19S fraction in the presence of detergent and/or reducing agent

Aliquots of the 19S fraction and the proteinase-K-treated 19S fraction were separated by one-dimensional PAGE on gels in the presence or absence of detergent. The 19S fraction, when separated on non-denaturing gels, contains two high molecular weight components (Figure 3.9, panel A, lanes 1 and 3), and the proteinase-K-treated fraction contains only the well-labelled M_r ~877,000 component (Figure 3.9, panel A, lanes 2 and 4) (see also Figure 3.6). When the 19S fraction and the proteinase-K-treated 19S fraction are separated by one-dimensional PAGE in the absence of detergent, but with β-MEt added to the sample prior to electrophoresis, their mobility is

Figure 3.8. Electron microscopic analysis of the proteinase-K-treated 19S fraction. Panel A, proteinase-K-treated 19S fraction (X 700,000). Panel B, proteinase-K-treated horse spleen ferritin (X 700,000). Scale bar = 20 nm. Samples were prepared by a one-step negative stain protocol. Treatment of the 19S fraction with proteinase-K eliminates the rectangular particle as well as the 10.5 nm particle, and only the 12-13 nm particle remains (see Figure 4 for comparison); and it is identical in size and appearance to horse spleen ferritin.


A
B

Figure 3.9. One-dimensional PAGE analysis of the 19S fraction in the presence of detergents. Aliquots of the 19S fraction and the proteinase-K-treated 19S fraction were separated by PAGE (5% separating gel) in the absence of detergent (Panel A), in the presence of Sarkosyl (Panel B), and in the presence of SDS (Panel C). Lanes 1 and 3, 19S fraction; lanes 2 and 4, proteinase-K-treated 19S fraction. Lanes 1 and 2, Coomassie-blue stain; lanes 3 and 4, fluorograms of lanes 1 and 2, respectively. Separation of the 19S fraction, and the proteinase-K-treated 19S fraction, in the presence of detergents only (i.e. no reducing agent) alters the mobility of the labelled, M_r ~877,000 component to M_r ~ 618,000. Marker proteins of relative molecular mass ($M_r \times 10^{-3}$) are indicated.


877
618
382
A
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1
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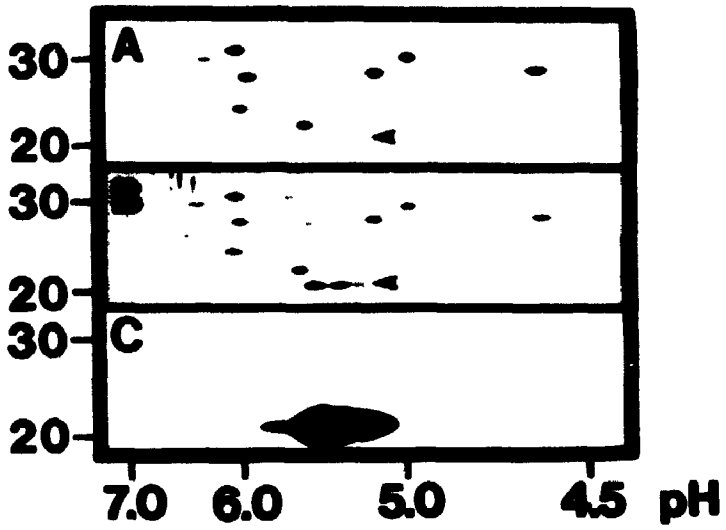
669
440
232
140

unaffected (not shown). However, when the 19S fraction and the proteinase-K-treated 19S fraction are separated by one-dimensional PAGE in the presence of Sarkosyl (Figure 3.9, Panel B) the M_r ~562,000 component appears to be more stable while the mobility of the M_r ~877,000 component changes to M_r ~618,000; the mobility of both components is unaffected by β-mercaptoethanol (not shown). When the 19S fraction and the proteinase-K-treated 19S fraction are separated in the presence of SDS (Figure 3.9, Panel C), the M_r ~562,000 component is denatured, and the mobility of the M_r ~877,000 component is altered to M_r ~618,000. Once again, β-mercaptoethanol has no effect on the mobility of either component (not shown). When the 19S fraction and the proteinase-K-treated 19S fraction are treated with RNase, however, there is no effect on the mobility of either component (not shown).

3.3.10 Two-dimensional PAGE analysis of the 19S fraction

Separation of the 19S fraction by two-dimensional PAGE (Figure 3.10, Panel A) reveals that the ~22-32 kDa set of proteins are distributed in a pattern similar to that of the prosome (Martins de Sa, et al., 1989) and the proteasome (Tanaka et al., 1989). Separation of trichloroacetic acid (TCA)-precipitated 19S fraction by two-dimensional PAGE (Figure 3.10, Panels B and C) reveals that the 450 kDa protein consists of protein subunits that are similar in size (21 kDa)

Figure 3.10. Two-dimensional PAGE analysis of the 19S fraction. Only the ~15--35 kDa region of the second dimension slab gels are shown. Panels A and B, Coomassie-blue stain. Panel A, 19S fraction. The ~22--32 kDa set of proteins are distributed over a wide range of pIs. Panel B, the proteins in the 19S fraction were precipitated by the addition of trichloroacetic acid (see Materials and Methods for details) prior to electrophoresis. This causes the ferritin polymer (>450 kDa) to separate into its 21 kDa subunits. These subunits (not seen in Panel A) are similar in size and pI to some of the ~22--32 kDa set of proteins. Panel C, fluorogram of panel B; only the 21 kDa subunits are labelled. Isoelectric points were estimated as described (see Materials and Methods).


30
A
20
30
B
20
30
C
20
7.0
6.0
5.0
4.5
pH

and pI to the prosome/proteasome subunit proteins.

3.3.11 Immunoassay of the 19S fraction using antibody to prosome protein P27

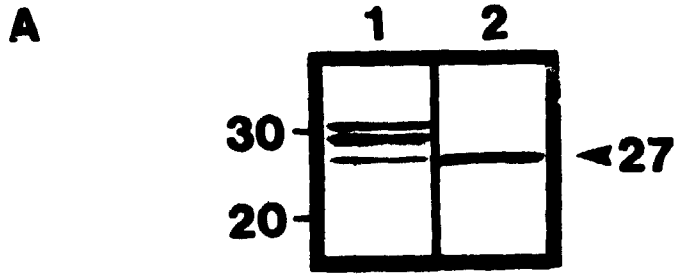
The 19S fraction was separated by one-dimensional SDS-PAGE and by two-dimensional IEF/SDS-PAGE, and the electrophoretically-separated proteins were transferred to nitrocellulose for immunoassay. One member of the ~22-32 kDa set of proteins cross-reacts with antibody to prosome protein P27 (Figure 3.11).

3.3.12 Protease assay of the 20S and 19S fractions

The 20S, 19S, and the proteinase-K-treated 19S fraction were assayed for protease activity by incubating aliquots in the presence of N-Succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin. The 20S fraction contains protease activity that is concentrated in the 19S fraction, and this activity is eliminated by treatment with proteinase-K (Figure 3.12).

The particles purified from the 19S fraction by electroelution were also assayed for protease activity *in vitro*. The results confirm that the protease activity that is present in the 19S fraction is associated with the M_r ~562,000 component, and suggests that this activity is stimulated by the addition of ATP, and by the addition of poly-L-lysine (Table 3.1).

Figure 3.11. Immunoblot analysis of the 19S fraction. Aliquots of the 19S fraction were separated by one-dimensional SDS-PAGE or by two-dimensional IEF-SDS-PAGE, electrophoretically transferred to nitrocellulose, and incubated with a monoclonal antibody to prosome protein P27. Panel A, one-dimensional SDS-PAGE separated 19S fraction. Lane 1, Coomassie-blue stain of the ~20--35 kDa region. Lane 2, immunostained blot of the region of Panel A1. Panel B, two-dimensional IEF-SDS-PAGE separated 19S fraction. Panel B1, Coomassie-blue stain of the ~20--35 kDa region of the second dimension slab gel. Panel B2, immunostained blot of the region of Panel B1. One of the members of the ~22--32 kDa set of proteins (denoted by an arrow in panel B1) crossreacts with antibody to prosome protein P27. Marker proteins of relative molecular mass ($M_r \times 10^{-3}$) are indicated. Isoelectric points were estimated as described (see Materials and Methods).


A
1
2
30
20
27

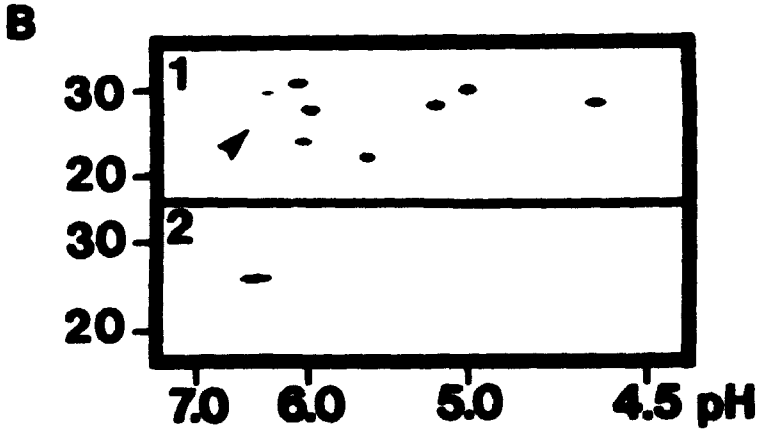

B
30
20
1
30
20
2
7.0
6.0
5.0
4.5 pH

Figure 3.12. Protease assay of the 20S, 19S, and proteinase-K-treated 19S fractions. Aliquots of the samples were assayed for protease activity by incubating them in 96-well microtiter plates in the presence of N-succinyl-leu-leu-val-tyr-7-amido-4-methylcoumarin. After incubation, plates were irradiated with ultraviolet light and photographed. Lanes 1 and 2, 5 μl sample volume; lanes 3 and 4, 20 μl sample volume. The protease activity of the 19S fraction (Row 2) has been concentrated from the 20S fraction (Row 1), and is eliminated by proteinase-K treatment (Row 3). C = control well (i.e. no sample, buffer only).

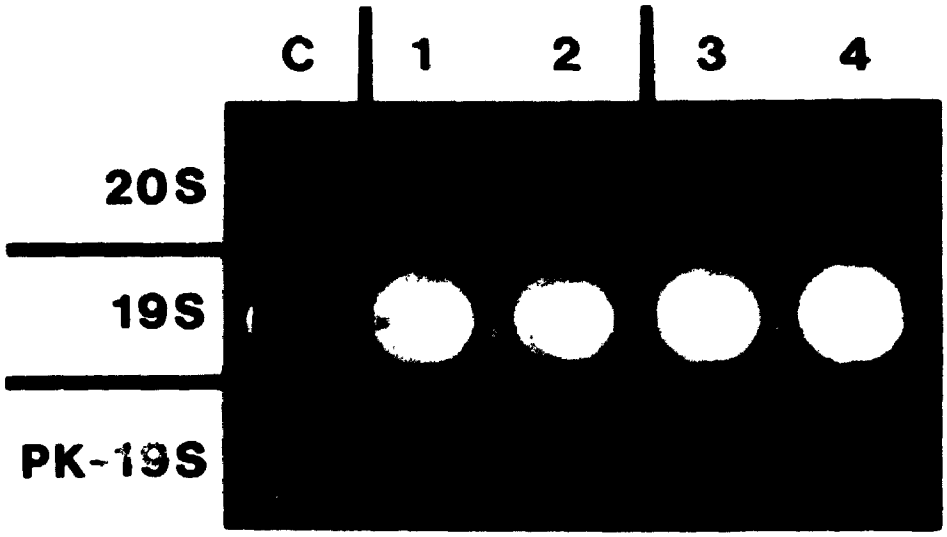

C
1
2
3
4
20S
19S
PK-19S

Table 3.1 Radioactive assay of proteolytic activity of purified 19S components (see Materials and Methods for details).

	Proteolytic activity (Units/μg protein)		
	No ATP or poly-L-lysine	+ ATP	+ poly-L-lysine
Component			
M_r ~877,000	0.000	0.000	0.000
M_r ~562,000	0.050	0.212	0.696

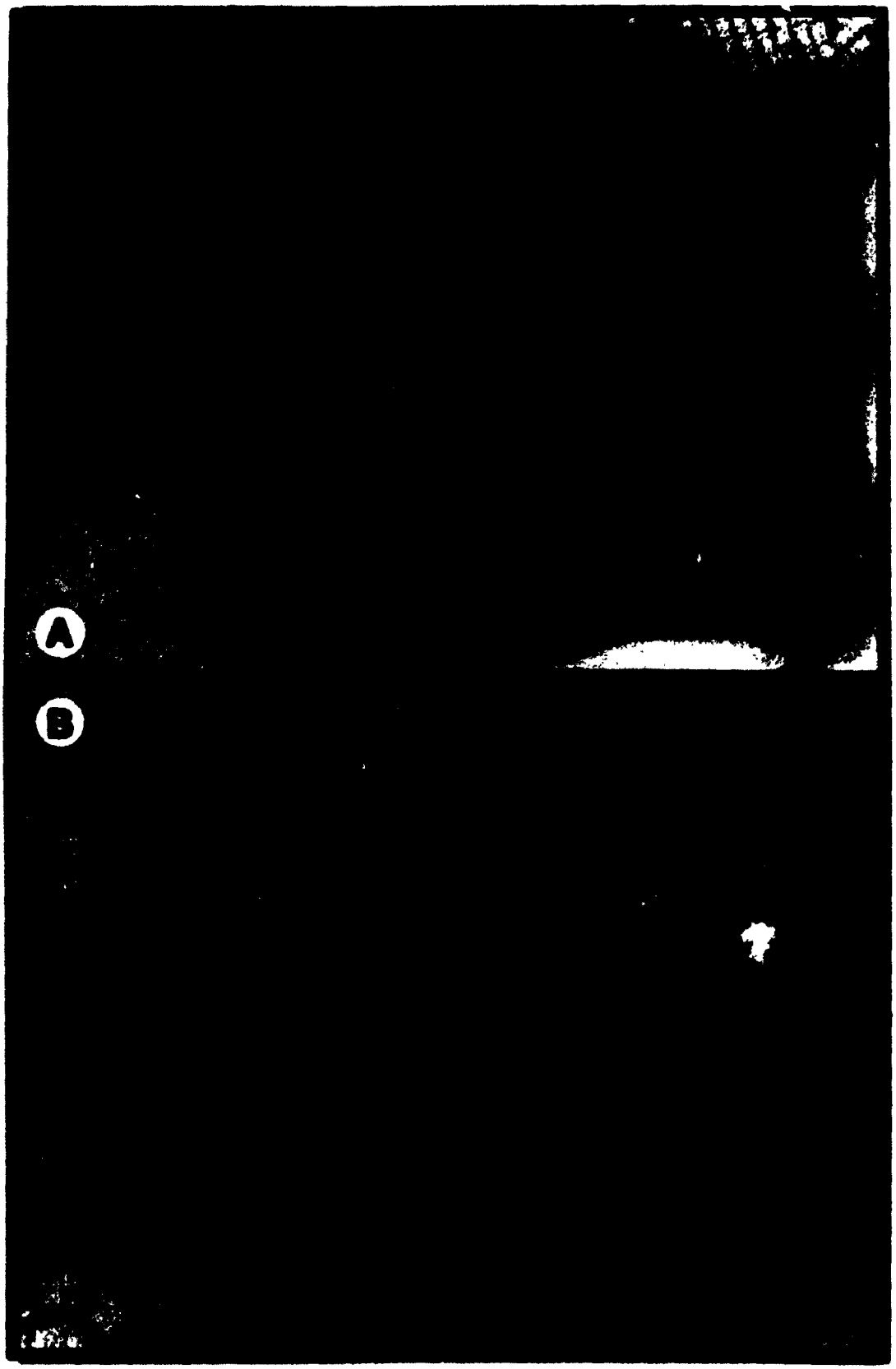
3.3.13 Electron microscopic examination of concentrated 19S fraction

Electron microscopic examination of concentrated 19S fraction (Figure 3.13, Panel A) reveals that the ratio of rectangular profiles to 10.5 nm particles has changed dramatically (compare to Figure 3.5), while the relative number of 12-13 nm particles has remained the same. Interestingly, while the ratio of the two particles has changed, they are not randomly distributed. Most of the rectangular profiles are together, and separate from most of the 10.5 nm particles; interspersed among each are some of the 12-13 nm particles. The 10.5 nm particles are also packed in an arrangement that suggests that they have 6-fold symmetry; in fact, they appear to be hexagonal (Figure 3.13, Panel B). It seems likely that the 10.5 nm particles are actually end-on views of the rectangular profile, and that this particle is therefore a 6-sided cylinder approximately 15 X 10 X 10 nm.

3.3.14 Image enhancement of the cylindrical particle

The side and end-on images of the cylindrical particle were enhanced by the superimposition of computer-recorded images. The cylindrical particle consists of a central body (~10 nm diameter) with two disk-like end caps that are

Figure 3.13. Electron microscopic examination of concentrated 19S fraction. Panel A, low magnification view of a negatively-stained whole mount (X 200,000). Panel B, high magnification view of a negatively-stained whole mount (X 700,000). Panel A. The number of 10.5 nm particles changes dramatically when the sample is concentrated (from 3-4% of the sample to ~50%), while the number of rectangular profiles decreases and the number of 12-13 nm particles remains the same (see arrowheads; compare to Figure 4); also, the 10.5 nm particles are not randomly distributed. The 10.5 nm particle is interpreted as the end-on view of the rectangular profile. Panel B. Two ferritin molecules (F) are distinguished by their greater size. The 10.5 nm particle appears to have a hexagonal profile (H). Both samples were prepared by a one-step stain protocol. Scale, bar = 20 nm.


A
B

2.5 nm thick (Figure 3.14, Panels A1, A2, and panel C). Enhancement of the end-on view confirms that the particle is hexagonal (Figure 3.14, Panels B1 and B2).

3.3.15 Electron microscopic analysis of one-step vs. two-step negative staining

Preparation of the 19S fraction for electron microscopy by a one-step staining protocol (see Materials and Methods) reveals that it consists mostly of cylindrical particles with a few particles of apoferritin (Figure 3.15, Panel A). However, if the sample is prepared by a two-step staining protocol, the sample contains round particles (~10-11 nm diameter) that resemble the prosome (see Schmid et al., 1984), and few cylinders (Figure 3.15, Panel B). The sample still contains the 12-13 nm particle. It appears that the cylinders degrade when the sample is prepared by the two-step protocol.

3.3.16 Analysis of the 35S fraction

The ~35S region of the SW28 gradient (fractions 11-13; see Figure 3.1) was concentrated by ultracentrifugation. The pellet was resuspended in HSB and separated on a high-salt sucrose gradient as previously described for the 20S sample. The ~35S region of the second sucrose gradient was collected,

Figure 3.14. Image enhancement of the cylindrical particle isolated from the 19S fraction of quail reticulocyte PRS. Panel A, image enhancement of the rectangular profile. Panel A1, unenhanced, computer-recorded image of a single particle. Panel A2, enhanced average image of 10 particles (a total of 20 images). This particle appears to consist of two 2.5 nm thick, disk-like ends with a somewhat spherical central component. Panel B, image enhancement of the end-on profile. Panel B1, unenhanced computer-recorded image of a single particle. Panel B2, enhanced, average image of 20 particles. The cylindrical particle has a hexagonal end-on profile. Panel C, schematic diagram of the enhanced image of panel A2. See Materials and Methods for details.

C

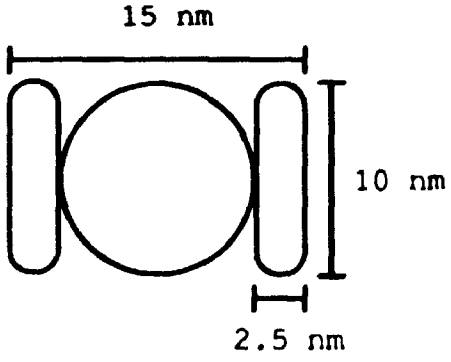
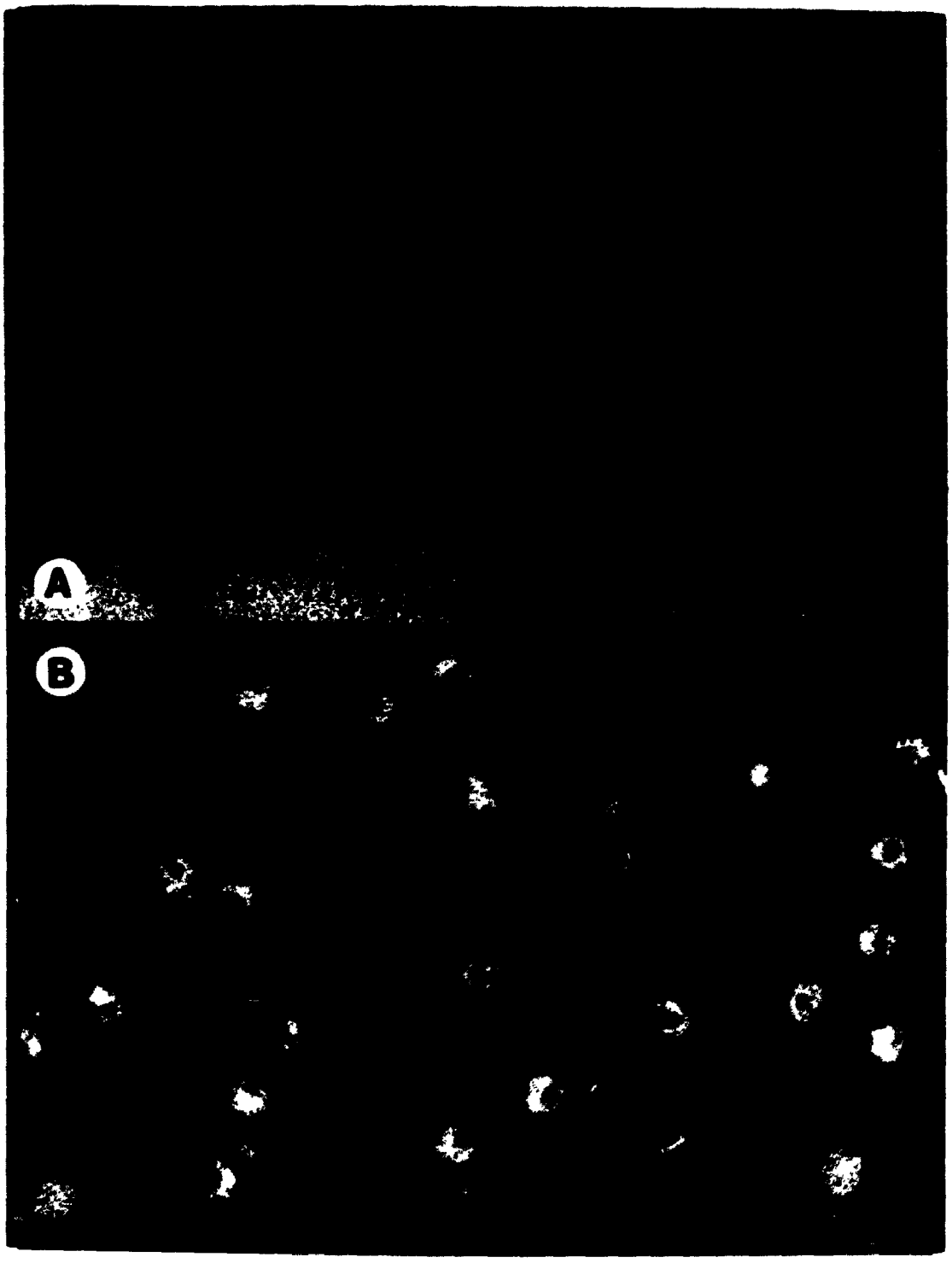


Figure 3.15. Electron microscopic comparison of the 19S fraction prepared by one-step versus two-step negative stain protocols. Panel A, sample prepared by one-step stain protocol. Panel B, sample prepared by two-step stain protocol. See Materials and Methods for details. When the sample is negatively stained using a two-step stain protocol, few cylindrical particles are found (1); only the 12-13 nm particle (2) and a ~10-11 nm round particle remain. This preparation resembles the prosome (see Schmid et al., 1984). Scale bar = 20 nm.


A
B

concentrated by ultracentrifugation, and resuspended in LSB. This fraction is referred to as the 35S fraction.

3.3.16.1 Electron microscopic analysis of the 35S fraction

Electron microscopic examination (Figure 3.16) shows that the 35S fraction contains a particle ~12 nm in diameter that is indistinguishable from quail ferritin isolated by treatment with proteinase-K (see Chapter 1 and Atkinson *et al*. (1989).

3.3.16.2 One-dimensional SDS-PAGE analysis

One-dimensional SDS-PAGE analysis shows that the 35S fraction contains a single protein component. This protein is ~450 kDa, is resistant to proteinase-K, contains iron, separates into 21 kDa subunits when boiled in the presence of β-mercaptoethanol and SDS prior to electrophoresis, and both the ~450 kDa protein and its 21 kDa subunits cross-react with antibodies to horse spleen ferritin (Figure 3.17, panel A).

One-dimensional PAGE analysis on 5% slab gels shows that the 35S fraction consists of an M_r ~877,000 component whose mobility is increased in the presence of SDS (Figure 3.17, panel B).

Figure 3.16. Electron microscopic appearance of negatively-stained 35S fraction. Panel A, 35S fraction. Panel B, quail ferritin. The particle present in the 35S fraction is identical in size and appearance to quail red blood cell ferritin isolated by the method of Atkinson et al. (1990). Scale bar = 20 nm.

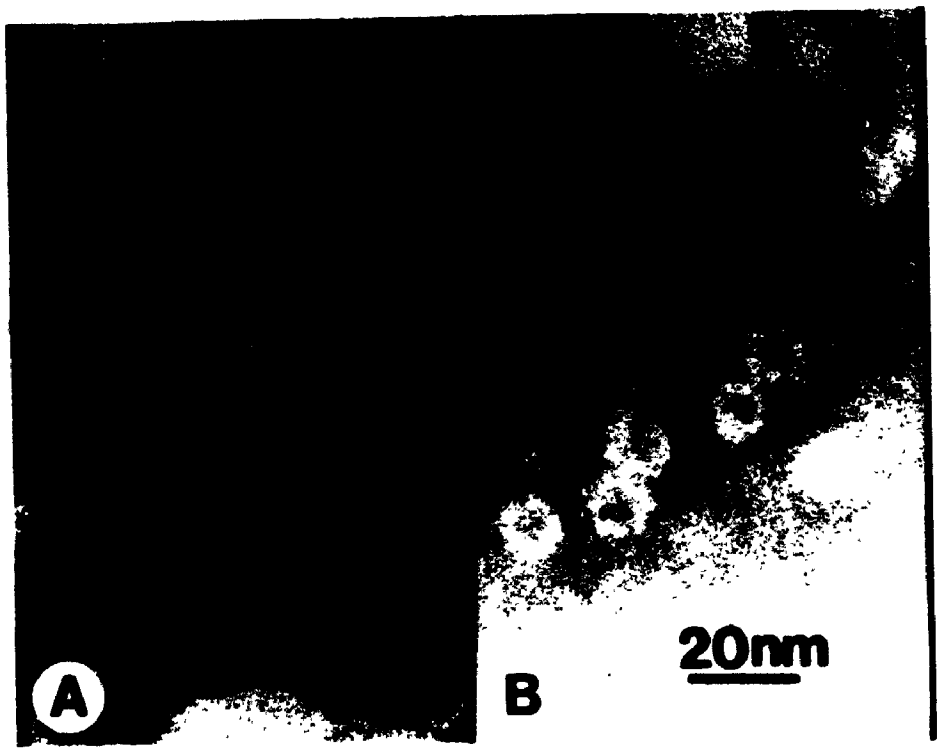
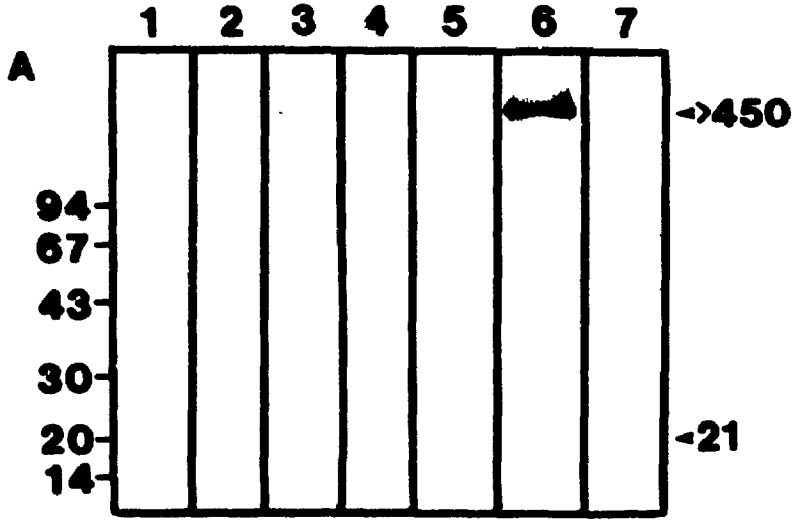

A
B
20nm

Figure 3.17. Electrophoretic analysis of the 35S fraction. Panel A, one-dimensional SDS-PAGE. Lanes 1 to 4, Coomassie blue-stained; lane 5, stained for iron; lanes 6 and 7, immunoblot assay. Lanes 1, 3, 5, and 6, sample directly applied to gel; lanes 2,4, and 7, sample heated in the presence of SDS and β-mercaptoethanol prior to electrophoresis. Lanes 1, 2, 5, 6, and 7, 35S fraction; lanes 3 and 4, proteinase-K-treated 35S fraction. Marker proteins (M_r x 10^{-3} are indicated). The 35S fraction contains a proteinase-K-resistant ~450 kDa iron-containing protein that separates into 21 kDa subunits. Both the ~450 kDa protein and the 21 kDa subunit crossreact with antibodies to horse ferritin. Panel B, fluorographic analysis on 5% slab gels. Lane 1, sample separated on a nondenaturing 5% gel; lane 2, sample separated on a 5% gel in the presence of SDS. The 35S fraction contains an M_r ~877,000 component that migrates at M_r ~618,000 in the presence of SDS. Pan 1 C, separation of RNA by agarose gel electrophoresis and hybridization of globin cDNA. Lane 1, ethidium bromide-stained RNA ladder (bases x 10^{-3}); lane 2, autoradiogram of RNA hybridization. RNA extracted from the 35S fraction was separated on a 1.4% agarose gel and probed with a globin cDNA (see Materials and Methods for details). The probe hybridized to RNA approximately 80 nucleotides in length. Panel D, separation of 35S fraction RNA by urea-PAGE. Lane 1, yeast tRNA (~80 nucleotides), lane 2, 35S fraction RNA. The RNA of the 35S fraction is approximately the same size as yeast tRNA.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94
67
43
30
20
14
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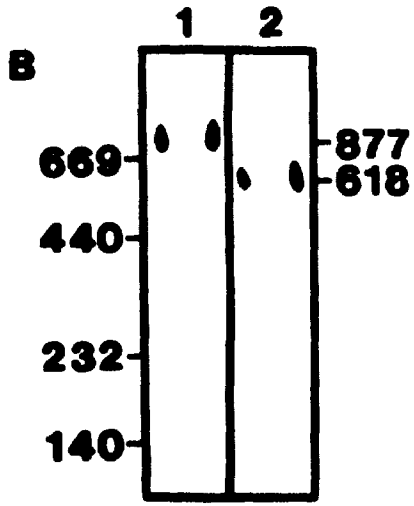
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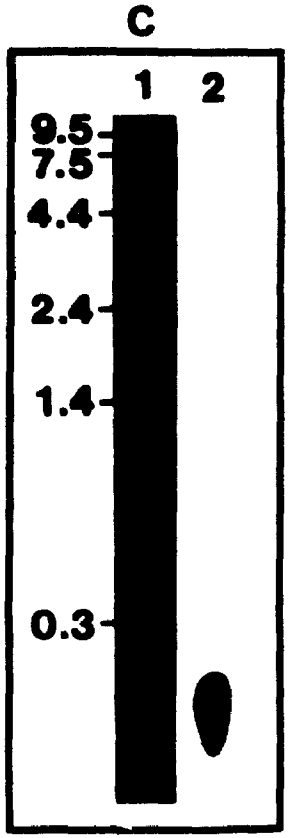
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7.5
4.4
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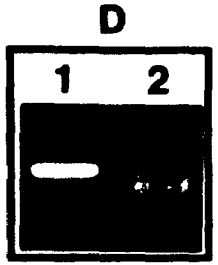
1.4
0.3


D
1 2

3.3.16.3 Analysis of RNA found in the 35S fraction

The 35S fraction was phenol-extracted and the RNA was separated by agarose gel electrophoresis and by urea/acrylamide gel electrophoresis. The agarose gel-separated RNA was transferred to nitrocellulose and probed with a ^{32}P-labelled globin cDNA. The 35S fraction contains RNA(s) ~80 nucleotides in length that hybridize with a globin cDNA (Figure 3.17, panel C).

3.4 DISCUSSION

The 20S fraction isolated from quail reticulocyte lysates by sucrose density gradient ultracentrifugation contains a large number of proteins ranging in size from ~14 kDa to ~450 kDa. The spectrum of proteins in the 20S fraction of quail reticulocytes is similar to that of the 20S fraction of duck reticulocytes (Vincent et al., 1980) with major components of ~90 kDa and ~48 kDa, a cluster of proteins at ~22--32 kDa, and many other minor components.

The 19S fraction contains a ~22--32 kDa set of proteins similar to the proteins described as components of the prosome (Schmid et al., 1984) and the proteasome (Tanaka et al., 1988a; Arrigo et al. 1988). These proteins incorporate little, if any, ^{35}S-methionine in quail reticulocytes.

The 19S fraction also contains a previously unreported >450 kDa protein that is very well-labelled with ^{35}S-methionine. Although this protein appears to be a contaminant from adjacent fractions of the sucrose gradient, when the 19S fraction is concentrated and reapplied to the same type of gradient from which it was collected, the >450 kDa protein is still found in the 19S region in the same relative proportions to the particle containing the prosome/proteasome protein subunits; it does not redistribute over the entire region of the gradient (not shown). Therefore, it seems that at least some of the >450 kDa protein might be part of a complex that

migrates at ~19S. This initially led to the conclusion that some of the >450 kDa protein found in quail reticulocytes is associated with the ~22--32 kDa set of proteins, and that it might be a core protein component of the "prosome".

3.4.1 The 19S fraction contains two distinct particles

The 20S fraction isolated from postribosomal supernatants of quail reticulocyte lysates by sucrose density gradient ultracentrifugation contains at least four, and perhaps as many as seven, large protein-containing components. The 19S "prosome" fraction, isolated from the high-salt-treated 20S fraction by sucrose density gradient ultracentrifugation, still contains two of the large protein-containing components of the 20S fraction (M_rs ~877,000 and ~562,000). Although both of these fractions have been reported to contain a single component (the 20S globin mRNP (Vincent et al., 1980) in the 20S fraction and the prosome in the 19S fraction (Schmid et al., 1984)), this does not appear to be the case.

Subsequent examination of the components of each band by SDS-PAGE (after concentration by electroelution) revealed that the major component of the 19S fraction (M_r ~562,000) consists of the ~22--32 kDa proteins, and the minor component (M_r ~877,000) contains the >450 kDa protein (not shown). Therefore, there are two distinct components in the 19S fraction, one of which (the M_r ~877,000 component) has not been detected

previously. It appears that the ~450 kDa protein is distinct from the prosome.

3.4.2 Biochemical characterization of the 19S fraction

3.4.2.1 Characterization of the M_r ~877,000 component

The M_r ~877,000 component of the 19S fraction contains a ~450 kDa protein. The size of this protein, and the fact that it migrates at 16S-19S, suggested that it might be ferritin. The protein was identified as ferritin by several different criteria: it is resistant to proteinase-K; it remains in the supernatant of heat denatured (70° C for 15 minutes) samples; it separates into 21 kDa subunits when boiled in the presence of SDS and β-mercaptoethanol prior to electrophoresis; and, both the ~450 kDa protein and its 21 kDa subunits crossreact with antibodies to ferritin.

3.4.2.2 The M_r ~877,000 component of the 19S fraction is a complex made up of ferritin and an unidentified component

Heat denaturation (70° C for 15 minutes) of both the 19S sample and the proteinase-K-treated 19S samples causes a change in the mobility of ferritin on nondenaturing gels (from M_r ~877,000 to M_r ~618,000). This appears to contradict the observation that proteinase-K-treated ferritin migrates at M_r

~600,000 on nondenaturing gels (see Chapter 1). However, those proteinase-K-treated samples had been subsequently heat-denatured to inactivate the enzyme. The heat-induced alteration in mobility of ferritin was duplicated by separating ferritin in the presence of detergents; β-mercaptoethanol did not affect the mobility of ferritin (not shown). Separation of the M_r ~618,000 component on SDS gels showed that it still contains the ~450 kDa protein (not shown). Therefore, it seems that the change in mobility caused by the heat treatment or detergent treatment must be due to the loss of another component. Since: 1) the ferritin does not contain any detectable iron (the loss of which might have altered its mobility); 2) β-mercaptoethanol does not alter the mobility of ferritin on native gels (not shown); and, 3) since it has been shown that proteinase-K-treated ferritin is free of non-ferritin protein contaminants (see Chapter 1), it seemed that there must be some other component attached to ferritin that causes it to migrate at M_r ~877,000, and that when this component is removed by heat denaturation, or by treatment with detergents, the ferritin migrates at M_r ~618,000. Having ruled out the presence of other proteins, the possible effect of the loss of iron, and the possibility that the band at M_r ~877,000 might have been a dimer, the remaining possibilities were that there is either RNA or lipid associated with the ferritin, and that this component is removed by treatments such as heat and detergents. The 19S fraction and the 19S

proteinase-K-treated fraction were incubated with RNase and no change in mobility was detected on nondenaturing gels (not shown). Therefore, it may be that the other component of the M_r ~877,000 particle is lipid, excluding the possiblity that any RNA present is resistant to the RNase.

3.4.2.2 Characterization of the M_r ~562,000 component

The M_r ~562,000 component of the 19S fraction has protease activity associated with it and contains a set of proteins with M_rs and pIs characteristic of the prosome/proteasome proteins. One of these proteins cross-reacts with a monoclonal antibody to prosome protein P27. Proteinase-K treatment of the 19S fraction destroys the M_r ~562,000 component, and the ~22--32 kDa set of proteins, and abolishes the protease activity. Therefore, I conclude that the M_r ~562,000 component of the 19S fraction is the prosome/proteasome.

3.4.3 Morphological characterization of the 19S fraction

Electron microscopic examination of the 19S fraction revealed that there might be as many as three morphologically distinct particles present. However, the 19S fraction contained only two biochemically distinct protein-containing particles, and it was necessary to determine which particle

corresponded to which electron microscopic image. One of the three profiles resembles the prosome, but it is clearly a minor component of the sample. The other minor component is a 12-13 nm round particle which is the same size as ferritin. The most common particle of the 19S fraction has a rectangular profile (~10 x 15 nm) and, in fact, resembles the proteasome (Arrigo et al., 1988). Although two reports (Falkenburg et al., 1988; Arrigo et al., 1988) concluded that the prosome and the proteasome are composed of the same protein subunits, there was no explanation for their different appearance by electron microscopy.

The resistance of ferritin to proteinase-K (see Chapter 1 and Atkinson et al., (1989)), allowed the determination of which image is that of ferritin: if the other particle(s) were susceptible to proteinase-K they would be eliminated and only the ferritin would remain. Electron microscopic examination of the proteinase-K-treated 19S fraction reveals that both the rectangular profile and the 10.5 nm profile are eliminated and only the 12-13 nm particle remains. This particle is indistinguishable in size and morphology from horse spleen ferritin, and so the 12-13 nm particle of the 19S fraction is ferritin (i.e. the M_r ~877,000 component of the 19S fraction). Therefore the rectangular image (and possibly the 10.5 nm image) is that of the M_r ~562,000 component.

It had been observed in samples where the particle concentration was greater than usual that there appeared to be

more of the 10.5 nm images, which suggested that this image might be an end-on view of a cylindrical particle. Electron microscopic examination of a 10X concentrated 19S sample shows that there are regions where the concentration of the 10.5 nm images has increased dramatically, and they are packed in hexagonal arrays. If there was simply an increase in the 10.5 nm particles then they would be expected to be distributed at random. Also, not only is the distribution unusually ordered, but the increase in the frequency of 10.5 nm particles is accompanied by a concomitant decrease in the number of rectangular profiles, while the frequency of 12-13 nm ferritin remains the same. Thus, it seems most likely that the 10.5 nm particle is an end-on view of the rectangular profile. Image enhancement of both views reveals that the particle is composed of three components: a 10 nm diameter central component enclosed by two hexagonal end caps.

Since ferritin is the 12-13 nm particle, and having shown that the other two images are different views of the same particle, the M_r ~562,000 particle of the 19S fraction is therefore a cylinder approximately 15 x 10 x 10 nm. This would account for the fact that there are only two detectable high molecular weight components on nondenaturing polyacrylamide gels.

The cylindrical particle of the 19S fraction is the proteasome. Its rectangular profile is identical in appearance to that reported by several authors. It is composed

of the same size class of subunits; and it possesses proteolytic activity. This lead to the conclusion that the 19S fraction isolated from quail reticulocytes contains two biochemically and morphologically distinct particles, the proteasome and ferritin; and that the prosome and the proteasome are the same particle. The problem still to be resolved is the description of the prosome as "round" and "raspberry-shaped", and the description, in some cases, of the proteasome as "ring-like".

3.4.4 Prosome particles are formed by degradation of the proteasome

The prosome has been characterized as a "round", "raspberry-like" particle with a "tendency to stack" (Schmid et al., 1984), and although the proteasome has been generally recognized as a cylindrical particle, it is often described as "ring-like" (Tanaka et al., 1988a). There has been virtually no discussion of the relevance of the two morphologies; the ring-shaped profile of the proteasome has been described as the end-on view and the cylindrical profile is thought to be a stack of rings (Kopp et al., 1986).

The difference in morphology is a result of the method of sample preparation for electron microscopy, and the "round" or "ring-like" particles are apparently the component parts of the entire particle. If the sample is prepared for electron

microscopy by a one-step stain protocol, it consists almost entirely of cylindrical particles (except for the ferritin that is present). However, if the sample is prepared for electron microscopy by a two-step stain protocol, where there is time for degradation to occur before fixation, there are few cylinders present, and the sample now consists of round profiles (some of which have a cleft) that resemble the prosome. There are two possible explanations: either the proteasome separates into its component parts, or it is predisposed to stand on end as a result of the two-step stain protocol. Of the two, separation and/or degradation into its component parts seems the most likely, particularly in view of its enhanced appearance as a particle consisting of three distinct components.

3.4.5 Ferritin is the core protein component of the prosome-like particle

The prosome-like particle was described by Akhayat et al. (1.87a) as a ~32S mRNP associated with repressed non-globin messenger RNAs. This particle resembled the prosome in morphology and size, and is a polymer consisting of a single 21 kDa proteinase-K-resistant protein subunit. It was associated with small RNAs (~80 nucleotides) that could not be dissociated by treatment with 0.5M KCl or SDS. These RNAs were shown

to hybridize to globin mRNA. This particle was also shown to inhibit translation of mRNA *in vitro* (including globin mRNA).

The 35S fraction isolated from quail reticulocytes contains a particle that is indistinguishable from quail ferritin by conventional means. The 35S fraction contains a ~450 kDa proteinase-K-resistant, iron-containing protein that cross-reacts with antibodies to horse ferritin. The 450 kDa protein is a constituent of an M_r ~877,000 component whose mobility is increased in the presence of SDS. Phenol extraction of the 35S region yields RNA(s) of ~80 nucleotides that hybridize with a globin cDNA. The sole protein constituent of the 35S fraction is ferritin, and I conclude that ferritin is the core protein component of the prosome-like-particle, and propose that ferritin may play a role in the repression of globin (and perhaps other) mRNAs.

4. SUMMARY

Ferritin can be isolated from quail reticulocytes by a rapid two-step purification method that gives high yields of pure, undegraded ferritin. Ferritin remains in the supernatant of heat-denatured samples, while most other proteins are denatured and can be pelleted. Subsequent treatment of the supernatant with proteinase-K eliminates any proteins that are not denatured by the heat treatment, and also removes proteins that may have been associated with ferritin *in vivo* or that become associated with ferritin as a result of the heat treatment. This procedure can be of use where the effect(s) of ferritin is/are being studied and a pure preparation is required.

Ferritin is a heat shock protein of quail reticulocytes. Its synthesis in heat-shocked cells is 3-5 times greater than in control cells. The isoforms of ferritin subunits synthesized in response to heat shock are identical in size and pI to isoforms made in control cells. The mechanism of this increased synthesis has been shown to involve translational control, i.e. there is little or no new transcription of ferritin mRNA, but rather increased translation. The heat shock-stimulated synthesis of ferritin may be mediated by the denaturation of hemoglobin (and subsequent release of iron) that occurs during heat shock.

Vincent et al. (1983) reported that Coomassie-blue staining of the proteins electrophoretically-separated from the 20S globin mRNP and 35S non-globin mRNP isolated from duck reticulocytes revealed that there was a 19 kDa proteinase-K-resistant protein associated with the 35S mRNP that could not be detected in the 20S mRNP. However, this 19 kDa protein was found among the labelled proteins in both the 20S and 35S mRNP fractions, and it migrated on two-dimensional gels to the same position, relative to the prosome proteins, as do the 21 kDa ferritin subunits of quail red blood cells (Chapter 3, Figure 10). Akhayat et al. (1987b) showed that the 19S prosome fraction contained a second particle with a predominant 21 kDa protein. However Akhayat et al. (1987b) described this particle as a 32S "prosome-like-particle" containing mRNA and composed primarily of a proteinase-K-resistant 21 kDa protein. They also reported that this particle inhibited translation, possibly by interacting with the mRNA. These reports suggest that an additional high molecular weight protein-containing component has been observed in the "prosome" fraction isolated by sucrose density gradient ultracentrifugation.

Ferritin is a consistent, albeit minor, constituent of the 19S "prosome" fraction. It is found as the protein component of an M_r ~877,000 particle. There are two possible explanations as to why ferritin has not been previously detected as a component of this fraction. First, the method used by Schmid et al. (1984) for recovering proteins from

sucrose gradient fractions was by trichloroacetic acid precipitation. This method separates ferritin into subunits which are similar in size (21 kDa) and pI to the smallest subunits of the prosome, and therefore it may have escaped notice. Secondly, much of the prosome work (especially that with avian (i.e. duck) red blood cell prosomes) was done with cells that were not radioactively labelled, and detection of a minor component such as ferritin is limited by conventional staining techniques.

Ferritin is also the protein component of the "prosome-like particle" described by Akhayat et al. (1987a). The 35S fraction contains a proteinase-K resistant, iron-containing ~450 kDa protein that separates into 21 kDa subunits, and which cross-reacts with antibodies to horse ferritin. This fraction also contains small RNAs that hybridize to a globin cDNA.

The electrophoretic mobility of ferritin on native gels is increased by heat or detergent treatment, suggesting that some component (eg. small RNAs) is being dissociated from ferritin. However, treatment of the ferritin with RNase does not alter its mobility on native gels. It is possible that these RNAs are nuclease resistant (as was shown by Dineva et al. (1989)), or perhaps some other component (eg. lipid) is being dissociated from the ferritin.

The major component of the 19S fraction is the proteasome: a M_r ~562,000 particle, composed of a ~22--32 kDa set of

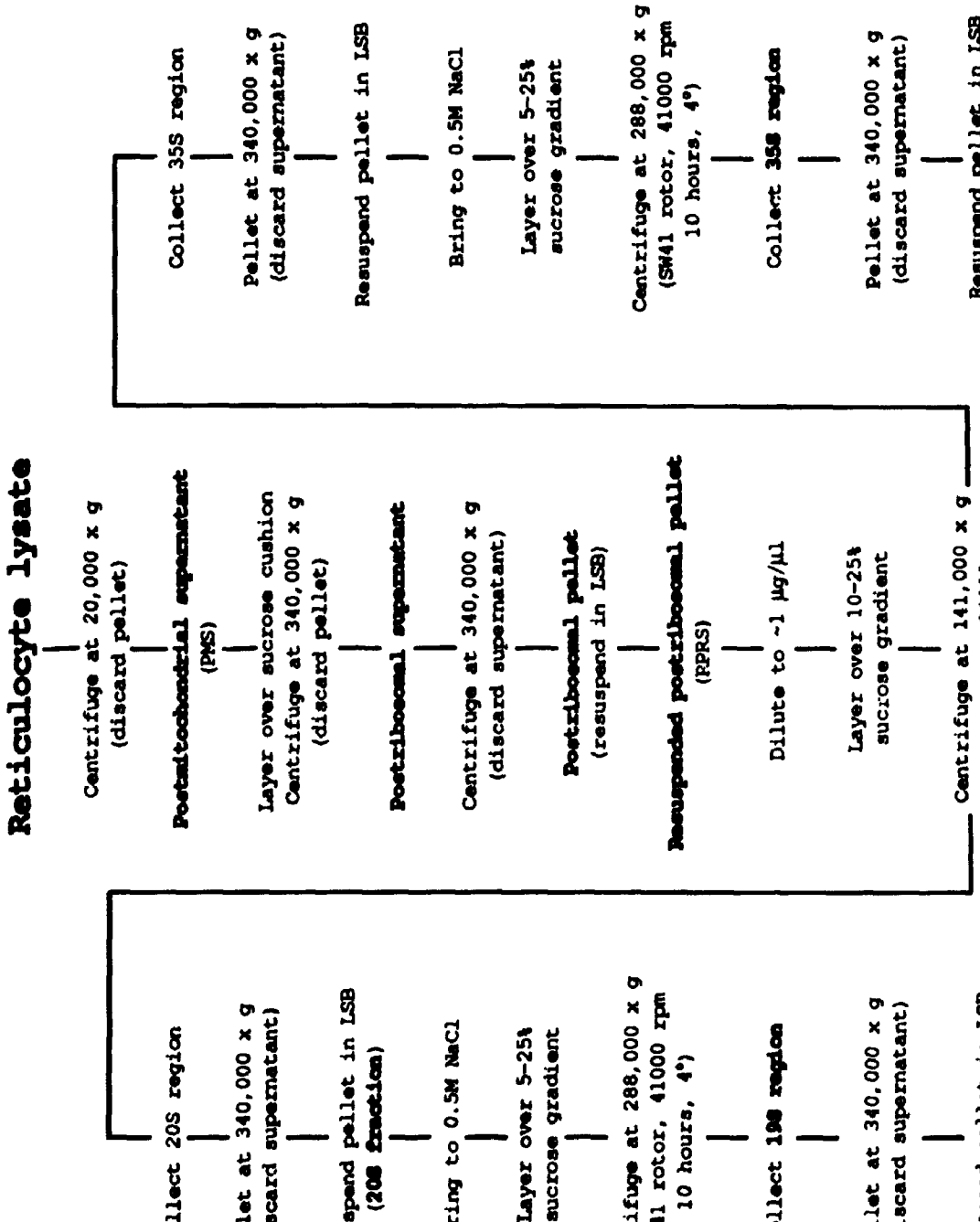
protein subunits. This particle has protease activity associated with it; and one of the subunits crossreacts with antibody to prosome protein P27. The proteasome is a cylinder (~15 x 10 x 10 nm) that appears to be composed of a central component approximately 10 nm in diameter enclosed by two flat hexagonal end caps.

The prosome particle is formed by degradation of the proteasome. The proteasome apparently separates into its component parts when prepared for electron microscopy by a two-step stain protocol. This accounts for the description of the prosome as "round" and "raspberry-like" showing a tendency to stack (Schmid et al., 1984; et seq.), and, in some cases, for the description of proteasomes as ring-like (Tanaka et al., 1988b).

I propose that ferritin may be associated with globin mRNA since: 1) small RNAs that hybridize to a globin cDNA can be extracted from fractions (~35S) that contain ferritin but which do not appear to contain any other protein (Chapter 3); 2) the "prosome-like-particle", which I have identified as ferritin, was reported to inhibit translation of globin mRNA possibly by associating with the mRNA itself (Akhayat et al., 1987a); and 3) ferritin was shown to inhibit globin synthesis (VanStone et al., 1966) although this was attributed by Freedman et al. (1976) to protease activity associated with ferritin rather than some interaction with mRNA. I hypothesize (as an extension of the proposal by Atkinson et al.

(1990) on the mechanism of ferritin induction in response to heat shock) that ferritin may function to regulate globin synthesis by sequestering its mRNA constitutively, and that during stresses such as heat shock, the increased availability of ferritin may account for the heat shock-induced decrease in globin synthesis by the increased sequestering of globin mRNA.

APPENDIX I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Postmitochondrial supernatant
(PMS)
Layer over sucrose cushion
Centrifuge at 340,000 x g
(discard pellet)
Postribosomal supernatant
Centrifuge at 340,000 x g
(discard supernatant)
Postribosomal pellet
(resuspend in LSB)
Resuspended postribosomal pellet
(PPRS)
Dilute to ~1 µg/µl
Layer over 10-25%
sucrose gradient
Centrifuge at 141,000 x g
(SW28 rotor, 28000 rpm
15 hours, 4°)
Collect 20S region
Pellet at 340,000 x g
(discard supernatant)
Resuspend pellet in LSB
(20S fraction)
Bring to 0.5M NaCl
Layer over 5-25%
sucrose gradient
Centrifuge at 288,000 x g
(SW41 rotor, 41000 rpm
10 hours, 4°)
Collect 19S region
Pellet at 340,000 x g
(discard supernatant)
Resuspend pellet in LSB
(19S fraction)
Collect 35S region
Pellet at 340,000 x g
(discard supernatant)
Resuspend pellet in LSB
(35S fraction)
Bring to 0.5M NaCl
Layer over 5-25%
sucrose gradient
Centrifuge at 288,000 x g
(SW41 rotor, 41000 rpm
10 hours, 4°)
Collect 35S region
Pellet at 340,000 x g
(discard supernatant)
Resuspend pellet in LSB
(35S fraction)

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